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Brit. J. Pharmacol. (1956), **11**, 351.

STUDIES IN THE CHEMOTHERAPY OF TUBERCULOSIS: ETHYL MERCAPTAN AND RELATED COMPOUNDS

BY

G. E. DAVIES, G. W. DRIVER, E. HOGGARTH, A. R. MARTIN, M. F. C. PAIGE, F. L. ROSE, AND B. R. WILSON

From the Imperial Chemical (Pharmaceuticals) Limited Research Department, Blackley, Manchester

(RECEIVED FEBRUARY 6, 1956)

Del Pianto (1950) claimed that a mixture of sodium 2-mercaptobenzthiazole-5-sulphonate and sodium *S*-ethylthiosulphate prevented the development of tuberculosis in infected guinea-pigs, and that neither compound alone showed antituberculous activity. The sodium 2-mercaptobenzthiazole-5-sulphonate could be replaced by other benzthiazoles and the sodium *S*-ethylthiosulphate by other alkylthiosulphates, but with the latter smaller "effects" were produced. Sodium *S*-ethylthiosulphate was tried because of its relation to cysteine, which had been found to potentiate the antibacterial activity of the benzthiazoles against *Staphylococcus aureus*.

While investigating this claim, we found that, though sodium 2-mercaptobenzthiazole-5-sulphonate was without antituberculous activity in infected mice or guinea-pigs, sodium *S*-ethylthiosulphate was very active.

The purpose of this communication is to report the development of this discovery. Since the completion of this work, the antituberculous activity of ethyl mercaptan compounds has been reported in the U.S.A. by two groups of workers (Brown, Matzuk, Becker, Conbene, Constantin, Solotorovsky, Winsten, Ironson, and Quastel, 1954; Kushner, Dalalian, Bach, Centola, Sanjurjo, and Williams, 1955), and, in addition, an abstract of a paper by Del Pianto (1953) has appeared in which he claims that, contrary to his earlier observations, sodium *S*-ethylthiosulphate is a highly potent antituberculous agent in experimental animals.

METHODS

Male albino mice weighing 18–22 g. were infected intravenously with 0.75 mg. wet weight of *Mycobacterium tuberculosis*, human type, strain 905, suspended in 0.1 ml. of distilled water. The suspension was made by ball-milling bacilli harvested from a 14-day culture on Löwenstein's medium. Compounds were administered orally twice daily, or subcutaneously once daily, to groups of 6 or 10 mice for two weeks. In each experiment a group of mice was treated with either streptomycin, 50 or 100 mg./kg., subcutaneously once daily, or isoniazid, 7.5 mg./kg., incorporated in powdered food.

With this type of infection untreated mice died with mean survival times ranging from about 18 to about 24 days. Streptomycin at 100 mg./kg. increased the mean survival time by about 9 days and isoniazid at 7.5 mg./kg. increased it by about 25 days.

RESULTS

Sodium *S*-ethylthiosulphate was the only active member of a series of sodium thiosulphates tested. The following *S*-substituted monosodiumthiosulphates were inactive: methyl-, *n*-propyl-, *iso*-propyl-, allyl-, *n*-amyl-, *n*-docedyl-, β-hydroxyethyl-, carbethoxymethyl-ethylene-bis-, trimethylene-bis-, benzyl-, phenyl-, *p*-chlorophenyl-, *p*-methoxyphenyl-, *p*-phenylenediamine-2-5-bis-. Sodium thiosulphate itself was also inactive.

Sodium *S*-ethylthiosulphate decomposed on storage to give diethyldisulphide which showed high antituberculous activity (Table I). The other disulphides shown in Table I were also effective,

TABLE I

DISULPHIDES ($R-S-S-C_2H_5$) ACTIVE AGAINST TUBERCULOSIS IN MICE

R	Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
C_2H_5—	20 p.o.	4·6	2·1
	2·5 s.c.	20·6	2·1
n-C_3H_7—	0·8 ,,	16·0	5·0
	0·4 ,,	5·0	5·0
Cl-C$_6$H$_4$— ..	0·6 ,,	13·0	5·0
	1·2 ,,	14·1	5·0
Cl_3C—	0·65 ,,	1·1	2·5
	1·3 ,,	9·4	2·7
—$CH_2CHNH_2.CO_2H$ ..	5·0 p.o.	6·3	2·1
	10·0 ,,	13·7	2·4

whereas dimethyl-, di-*n*-propyl-, di-*iso*propyl-, didodecyl-, di-carboxymethyl-, di-*p*-nitrophenyl-, and di-3:4-diaminophenyl-disulphides were inactive.

Since sodium *S*-ethylthiosulphate and the active disulphides could be decomposed *in vitro*, and presumably *in vivo*, to give ethyl mercaptan, we tested this substance and found it to be active in mice (Table II). The following related thiols were inactive: *n*-propane-, *n*-butane-, *iso*butane-, *n*-heptane-, β-aminoethane-, β-diethylaminoethane-, β-(1-piperidyl) ethane-, β-(4-morpholino) ethane-, β-anilinoethane-, *N*:*N*-anilinobis-β:β′-ethane-, *di*-β-hydroxyethane-, β-carboxymethane-, butane-1:4-di-, ethane-1:2-di-, *cyclo*hexane-, benzene-, 4-diethylamino-2-aminobenzene-, 4-hydroxypyrimidine-2-, 4-methyl-2-aminopyrimidine-4-, 2-phenyl-4-hydroxy-1:3:5-triazine-6-, 2-amino-4-hydroxylamino-1:3:5-triazine-6-, 2-hydroxy-1:3:5-triazine-4:6-di-, 4-nitrobenzimidazole-2-thiols; DL-penicillamine, DL-penicillamine ethyl ester, 2:3-dimercaptopropanol (BAL).

TABLE II

EFFECT OF ETHYL MERCAPTAN ON TUBERCULOSIS IN MICE

Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
5 p.o. 4 times daily	6·0	2·1
10 s.c. daily for 15 days ..	32·8	6·7
60 in drinking water for 15 days	42·2	6·7

We had thus established that ethyl mercaptan, or a source of ethyl mercaptan, was antituberculous in mice and that this property was peculiar to the ethyl homologue. Yet, as is shown below, the mercaptan had only a slight action on tubercle bacilli *in vitro*. These observations, taken in conjunction with the known chemical reactivity of thiols, suggested that a metabolite of ethyl mercaptan was the ultimate active agent. Two possible mechanisms of the metabolism of ethyl mercaptan were envisaged: (*a*) oxidative, and (*b*) that the thiol may act as an ethylating or "ethylthiolating" agent.

Oxidative Metabolism.—This possibility includes compounds formed by oxidation at the sulphur atom (which may have undergone prior methylation) giving sulphoxides, sulphones, sulphinic and sulphonic acids; compounds formed by oxidation at the 2-carbon atom, giving derivatives of ethanol, acetaldehyde or acetic acid, and compounds formed by oxidation at both these centres. Accordingly the following substances were tested: ethyl

TABLE III

EFFECT OF ETHYL THIOL-ESTERS OF CARBOXYLIC ACIDS ON TUBERCULOSIS IN MICE

Acid	Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
Acetic	20 p.o.†	4·6	2·6
Propionic	0·35 s.c.	1·3	1·7
	0·7 ,,	8·6	1·7
n-Butyric	0·35 ,,	3·3	1·7
	0·7 ,,	8·2	1·7
n-Caproic	0·5 ,,	3·1	1·7
	1·0 ,,	23·3	1·7
Lauric	0·75 ,,	2·7	1·7
	1·5 ,,	1·1	1·7
Dimethylaminoacetic	5·0 p.o.	6·3	2·3
	10·0 ,,	11·0	2·3
Malonic (bis ester)	5·0 ,,	7·0	1·8
	10·0 ,,	13·4	4·6
,, (mono ester)	0·45 s.c.	0·1	2·3
	0·9 ,,	0	2·4
Hippuric	5·0 p.o.	5·0	2·5
	10·0 ,,	7·6	2·3
Phenylacetic	1·0 s.c.	32·8	
	5·0 ,,	35·1	
	5·0 p.o.	10·3	2·0
	10·0 ,,	12·3	2·0
Cinnamic	1·0 s.c.	−3·6	5·0*
	2·0 ,,	−1·3	5·0
Benzoic	1·0 ,,	10·0	2·6
	1·0 ,,	40·4	12·0*
	8·0 p.o.†	9·3	3·1
Acetylsalicylic	1·0 s.c.	1·5	2·5
	10·0 ,,	14·7	2·5
Phthalic	4·0 p.o.†	2·2	3·1
	8·0 ,,†	8·1	3·2
m-Nitrobenzoic	8·0 ,,†	7·2	3·1
	20·0 ,,†	37·4	3·1
m-Aminobenzoic	1·0 s.c.	16·0	12·0*
iso-Phthalic	10·0 p.o.	46·7	
	10·0 s.c.	>83·0	
	5·0 ,,	58·4	
p-Toluic	8·0 p.o.†	13·1	3·2
	20·0 ,,†	25·7	3·1
p-Anisic	8·0 ,,†	6·1	3·1
	20·0 ,,†	28·4	3·1
p-Nitrobenzoic	2·0 ,,	3·4	5·9
	4·0 ,,	16·2	5·9
p-Aminobenzoic	1·0 s.c.	0·7	1·8
	2·0 p.o.†	1·7	2·1
	4·0 ,,†	2·0	2·1
p-Dimethylaminobenzoic ..	2·5 ,,	5·7	4·4
	5·0 ,,	8·3	8·3
	5·0 ,,	22·2	12·0*
p-Ethanesulphonylbenzoic ..	8·0 ,,†	4·7	2·5
	20·0 ,,†	12·7	2·5
p-Aminosulphonylbenzoic ..	0·75 ,,	2·3	2·3
	1·5 ,,	4·0	2·3
*Tere*phthalic	0·7 s.c.	7·8	2·6
	1·5 ,,	10·5	2·8
	20·0 p.o.†	10·4	2·0

TABLE III—*continued*

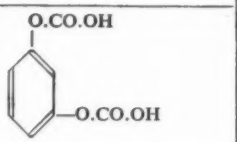
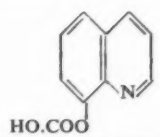
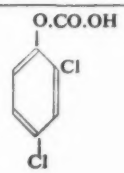
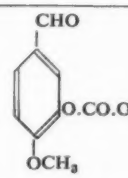
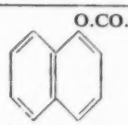
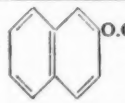
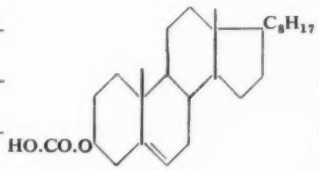
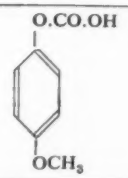
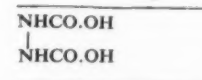
Acid	Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
Terephthalic (mono ester) ..	20·0 p.o.† 8·0 ,,†	0 0	1·8 1·8
2:4-Dichloro-benzoic	1·5 s.c.	7·3	2·6
iso-Nicotinic	1·0 ,, 10·0 ,, 8·0 p.o.† 20·0 ,,†	1·5 14·7 15·3 17·8	2·5 2·5 5·3 4·7
Carbonic	1·0 s.c. 2·0 ,,	10·6 10·6	1·7 1·7
$CH_3O.CO.OH$	0·35 ,, 0·7 ,,	2·9 2·8	2·3 2·3
$C_2H_5O.CO.OH$	2·0 p.o.† 4·0 ,,†	−0·7 5·8	2·7 2·7
n-$C_4H_9O.CO.OH$	0·5 s.c. 1·0 ,,	1·8 23·3	2·3 2·3
tert.-$C_4H_9O.CO.OH$	0·5 ,, 1·0 ,,	1·2 17·0	2·4 2·4
$C_2H_5O.CH_2CH_2O.COOH$ ..	0·55 ,, 1·1 ,,	2·1 6·4	2·3 2·3
$C_6H_{11}O.CO.OH$	0·55 ,, 1·1 ,,	1·5 10·8	2·3 2·3
$C_6H_5O.CO.OH$	0·55 ,, 1·1 ,,	6·4 23·4	2·3 2·3
	0·43 ,, 0·86 ,,	1·0 7·2	2·3 2·3
Carbamic	1·0 p.o. 2·0 ,,	2·7 4·6	1·7 1·7
Diethylcarbamic	1·0 ,, 2·0 ,,	−0·5 0·2	2·3 2·3
.. ..	0·5 s.c. 1·0 ,,	1·3 1·0	1·7 1·7
$O_2NC(CH_2O.CO.OH)_3$	1·5 p.o. 3·0 ,,	3·1 4·1	2·3 2·3
$C(CH_2O.CO.OH)_4$	5·0 ,, 10·0 ,,	1·7 4·4	1·9 1·8
	5·0 ,, 10·0 ,,	3·3 5·8	2·2 2·3
	0·7 s.c. 1·4 ,,	0·3 4·4	1·8 1·8

TABLE III—*continued*

Acid	Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
	12 p.o. 20 ,,	3·8 5·6	2·5 2·5
	0·7 s.c. 1·4 ,,	0·8 11·1	1·9 1·9
	5·0 p.o. 10·0 ,,	6·3 14·8	2·2 2·2
	5·0 ,, 10·0 ,,	−1·2 −1·4	1·9 1·9
	0·65 s.c. 1·3 ,,	6·4 12·0	2·0 2·0
	1·0 p.o. 2·0 ,,	3·1 8·4	2·1 2·1

* Treatment delayed until 14 days after infection.
† Daily dose administered in food.

methyl sulphide, sulphoxide and sulphone ; diethylsulphide, sulphoxide and sulphone ; ethanesulphinic acid ethyl ester, *n*-butyl ester, *N*:*N*-diethylamide, *N*:*N*-dibutylamide, and piperidide ; ethanesulphonic acid ; trithio-acetaldehyde (α and β forms) ; β-hydroxyethanethiol ; β-hydroxyethylmethylsulphide and sulphone ; β-hydroxydiethylsulphide and sulphone ; bis-β-hydroxyethylsulphide and sulphoxide ; thioglycollic acid and amide ; S-methylthioglycollic acid ; thioacetic acid ; S-ethylethanethiosulphinic acid, S-ethylethanethiosulphonic acid and butane-1 : 4-dithiol. They were all without therapeutic effect.

Ethylation.—The possibility that ethyl mercaptan may act as an ethylating agent *in vivo* was considered by examining the ethyl analogues of

choline, methionine and betaine. Other possible ethylating agents, such as diethylsulphide, diethylsulphate, ethylsulphuric acid and ethylxanthate were included, but none of these substances was antituberculous. In addition, thioethanolamine and compounds related to it (and hence to Coenzyme A), such as ethanolamine, bis-(β-aminoethyl) disulphide, bis-(β-aminoethyl) sulphide and sulphone, methyl-β-aminoethyl sulphide, and bis-(β-diethylaminoethyl)-sulphides; and the vinylsulphones, vinyl methyl sulphone, divinyl sulphone and reaction products of these with the amines glycine, ethylamine, cysteine and *p*-aminobenzoic acid, were all examined as potential antituberculous agents derived from ethyl mercaptan. These compounds, also, were inactive.

It was concluded from this preliminary work, that a therapeutically active compound in this series would have to be a source of ethyl mercaptan. Because of the unpleasant smell of ethyl mercaptan it was desirable that a therapeutically acceptable compound should release the thiol only after absorption. The class of compounds most likely to supply a drug acceptable both pharmacologically and pharmaceutically seemed to be the thiol-esters. The first of these to be prepared, ethyl thiolbenzoate, showed outstanding activity, a result which led to the examination of a large number of compounds of this type. It will be seen from Tables III and IV that the specificity of the ethyl group was demonstrated yet again. The thiol-esters were invariably more active when administered parenterally than when administered orally.

Other potential sources of ethyl mercaptan have been examined (Table V), but no compounds superior to the thiol-esters have been discovered.

Experiments in vitro

Ethyl mercaptan inhibited the growth of tubercle bacilli in protein-free liquid media at a concentration of 1:20,000. The addition of 0.5% bovine plasma albumin increased the inhibitory concentration to 1:5,000, and in the presence of 10% unheated horse serum there was no inhibition at 1:5,000. Ethyl thiolbenzoate and diethyl dithiol-*iso*phthalate, both of which are highly active in mice, were virtually inactive *in vitro*. Sera from animals receiving high doses of active thiol-esters were without bacteriostatic activity when diluted 1:10 in liquid media, as were small pieces of liver, lung, spleen, and kidney.

TABLE IV

THIOL-ESTERS AND RELATED COMPOUNDS INACTIVE AGAINST TUBERCULOSIS IN MICE

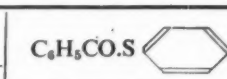
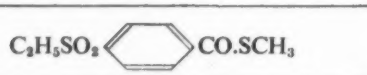
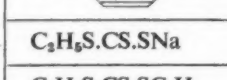
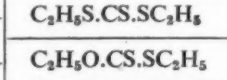
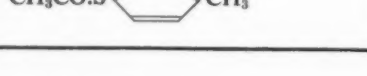
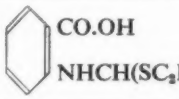
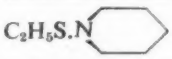
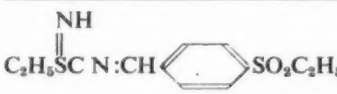
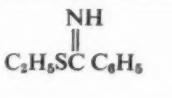
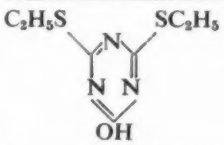
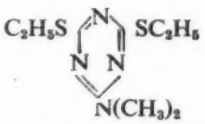
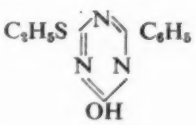
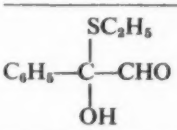
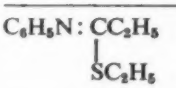
$CH_3CO.SCH_3$		$P(SC_2H_5)_3$
$C_6H_5CO.SCH_3$		$OP(SC_2H_5)_3$
		
$CH_3CO.SC_3H_7(n)$	$C_2H_5S.CS.SNa$	$C_6H_5SO_2.SC_6H_5$
$C_6H_5CO.SC_3H_7(n)$	$C_2H_5S.CS.SC_2H_5$	$C_2H_5SO.SC_2H_5$
$C_6H_5CO.SC_3H_7(iso)$	$C_2H_5O.CS.SC_2H_5$	$C_3H_7SO.SC_3H_7(n)$
$CH_2S.CO.CH_3$ $CH_2S.CO.CH_3$	$(C_2H_5)_2NCS.SC_2H_5$	$CH(SC_2H_5)_3$
	$NH_2NHCS.SC_2H_5$	$C(SCH_3)_4$
$CH_2S.CO.C_6H_5$ $CH_2S.CO.C_6H_5$	$C_6H_5CH:NNHCS.SC_2H_5$	$C(SC_2H_5)_4$
	$C_6H_{12}O_5:NNHCS.SC_2H_5$	C_2H_5SCN
$CH_3CO.SC_6H_5$	$C_2H_5SO_2.SC_2H_5$	$C_2H_5N=C(SC_2H_5)_2$
$C_6H_5CO.SC_6H_5$	$C_6H_5SO_2.SC_2H_5$	$C_6H_5CS.OC_2H_5$
		$C_6H_5CO.OC_2H_5$

TABLE V

EFFECT OF VARIOUS SOURCES OF ETHYL MERCAPTAN ON TUBERCULOSIS IN THE MOUSE

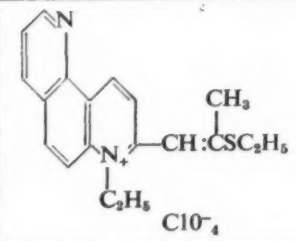
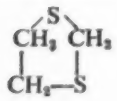
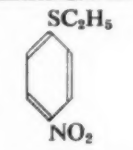
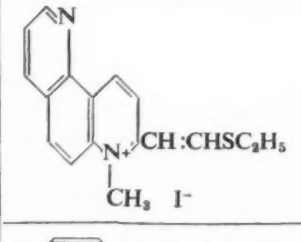
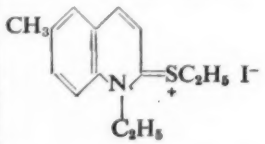
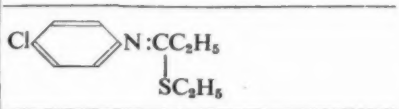
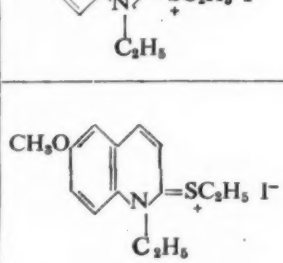
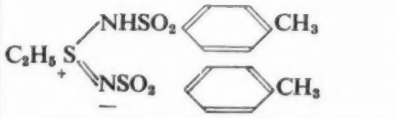
A. Active Compounds

	Dose (mg./20 g.)	Increased Mean Survival Time (Days)	Increase Required for Significance (Days)
$C_2H_5SCH_2CHNH_2.CO.OH$	4·0 p.o. 6·0 ,, 10·0 ,, 5·0 s.c.	3·2 0·1 7·8 −0·2	5.6 2·1 5·6
	5·0 p.o. 10·0 ,, 0·9 s.c.	3·8 5·8 2·2	1·8 1·8 2·0
$C_6H_5CH(SC_2H_5)CH(COCH_3)_2$	5·0 p.o. 10·0 ,,	1·4 2·6	1·8 1·9
	0·45 s.c. 0·9 ,,	1·6 6·1	2·6 2·6
	1·0 ,, 2·0 ,,	6·6 9·0	3·3 3·7
	8·0 p.o.* 20·0 ,, *	2·2 7·7	2·1 5·9
	2·5 ,, 5·0 ,,	1·9 3·9	2·7 2·7
	8·0 ,, * 20·0 ,, *	8·7 −0·8	6·7 6·7
	2·5 ,, 5·0 ,,	0·2 5·3	1·8 4·4
	2·0 ,, 5·0 ,,	2·1 7·4	1·8 1·8
	1·0 ,, 2·5 ,,	0 5·1	2·4 2·4

* Daily dose administered in food.

TABLE V—*continued*

B. Inactive Compounds

$CH_2(SC_2H_5)_2$	$CH_3COCH(CHSC_2H_5.C_6H_5)CO_2C_2H_5$	
$CH_3CH(SC_2H_5)_2$	$C_6H_5CHSC_2H_5.CH(CO_2C_2H_5)_2$	
$C_6H_{12}O_6$: $(SC_2H_5)_2$ D. Glucose diethyl acetal	$(C_6H_5CH(SC_2H_5)CH_2)_2CO$	
		
$(CH_3)_2C(SC_2H_5)_2$		
$CH_3CH_2OCH_2CH_2OCH_2SC_2H_5$		
$C_6H_5CH:CHCH(SC_2H_5).CH_2COC_6H_5$		

DISCUSSION

It is apparent from the foregoing results that ethyl mercaptan exerts a profound effect on the tuberculous process in infected mice. Any compound which can be expected to produce ethyl mercaptan either before or after absorption shows antituberculous activity, and this activity is specific for the ethyl radical, even closely related thiols being completely inactive. The antituberculous activity of ethyl mercaptan derivatives varies considerably, no doubt owing to variations in absorption and degradation, but the most attractive and consistently active group of derivatives consists of the ethyl thiol-esters. Further study has therefore been confined to these esters, and work on this topic will be reported separately.

SUMMARY

1. Ethyl mercaptan shows high antituberculous activity in the infected mouse.

2. No other thiols tested showed this activity.

3. Derivatives of ethyl mercaptan which can be metabolized to the parent substance are also active.

4. As potential drugs for the treatment of the human disease, the ethyl thiol-esters appear to be the most promising.

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Del Pianto, E. (1950). *Ric. Sci.*, **20**, 83.

—— (1953). Ibid., **23**, 1785. Cited in *Chem. Abstr.*, 1954, **48**, 13989.

Kushner, S., Dalalian, H., Bach, F. L., Centola, D., Sanjurjo, J. L., and Williams, J. H. (1955). *J. Amer. chem. Soc.*, **77**, 1152.

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INHIBITION OF HISTAMINE RELEASE BY SODIUM SALICYLATE AND OTHER COMPOUNDS

BY

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There are few reports in the literature of drugs which protect against allergic reactions by mechanisms other than antagonism to released humoral agents. Substances which inhibited the chain of reactions leading to release of active substances such as histamine might be useful in the treatment of conditions, suspected to be allergic in origin, that do not respond to treatment with antihistaminic drugs. Knowledge of the mode of action of such inhibitors of the allergic reaction, even if weak, might be useful in suggesting possible structures for more effective agents.

Sodium oxalate, sodium citrate, and phenol, completely prevent *in vitro* anaphylactic histamine release in rabbit blood; heparin is partially effective (Dragstedt, Wells, and Rocha e Silva, 1942; McIntire, Roth, and Richards, 1949). There is little agreement, however, on the ability of heparin to protect against acute anaphylactic shock *in vivo*; sodium citrate does reduce the amount of histamine liberated during anaphylactic and anaphylactoid reactions in the dog (Rocha e Silva, Scroggie, Fidlar, and Jaques, 1947; Rocha e Silva, 1950). Sodium salicylate inhibits anaphylactic histamine release from guinea-pig lung *in vitro* (Trethewie, 1951; Ungar and Damgaard, 1955) and modifies the response of isolated guinea-pig ileum to the specific antigen (Gray, Pedrick, and Winne, 1951); both sodium salicylate and acetylsalicylic acid protect rabbits against acute anaphylactic shock (Lepper, Caldwell, Smith, and Miller, 1950) although the latter is not effective against histamine shock (Campbell, 1948). Sodium salicylate also gives some protection against the passive reversed Arthus reaction in rabbits and guinea-pigs (Smith and Humphrey, 1949) and against the Schwartzman reaction (Schwartzman, Schneierson and Soffer, 1950).

This paper is mainly concerned with a study of the effect of sodium salicylate on various reactions in which histamine is released, in an attempt to throw some light on its mode of action.

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METHODS

Sensitization of Rabbits.—Rabbits were sensitized by subcutaneous injections of a modified Freund type antigen (protein of Wellcome normal horse serum 5% w/v in 0.85% w/v NaCl, 5 g.; ointment of wool alcohols B.P., 5 g.; liquid paraffin containing heat-killed avian tubercle bacilli 2 mg./ml., 10 g.) (Freund, 1948). Each animal received 1 ml. at two different sites on the first day. This was repeated once between days 7 and 14 and then again 10 days later. Boosting doses (2 by 1 ml.) were given when necessary. Seven days were allowed to elapse after an injection before blood samples were withdrawn.

General Methods.—The collection of blood, the biological assay of histamine, and the polysaccharides used have been described previously (Haining, 1955). Plasma histamine was usually extracted by the method of McIntire, Roth, and Shaw (1947), but sometimes, including all experiments where estimates of cellular histamine were required, Code's (1937) modification of the method of Barsoum and Gaddum was employed. Antigen-antibody precipitate was obtained by incubating serum from a sensitized rabbit with a small quantity of horse-serum for 30 min. at 37.5° C. The precipitate was washed 3 times with, and suspended in, a volume of 0.85% NaCl equal to that of the original serum. When activated plasma was required the plasma of dilute heparinized blood (heparin 1.5 units/ml.) was incubated with antigen-antibody precipitate for 1 hr. at 37.5° C. and then centrifuged to remove the precipitate. Anaphylatoxins were prepared by the method of Bordet (1913), and were tested on isolated guinea-pig ileum. Concentrations of activators referred to in the text are those present in the final preparation.

Inhibition of Histamine Release in Rabbit Blood.—Samples (6 ml.) of dilute heparinized blood (heparin 1.5 units/ml.) were mixed with 1 ml. of 0.85% w/v NaCl with and without inhibitor. The samples were incubated at 37.5° C. for 15 min.; 0.5 ml. of 0.85% w/v NaCl or the same solution containing Wellcome No. 2 horse serum 6% v/v was then added and the incubation continued for a further 15 min. The samples were chilled in ice and centrifuged at 3,000 rev./min. for 20 min. at room temp. Aliquots of the supernatant plasma were extracted and assayed for histamine.

Results

When blood from a sensitized rabbit was incubated with the specific antigen there was almost always a marked increase in the concentration of plasma histamine. (In 21 experiments, the mean plasma histamine of control samples was 0.6 μg./ml. and after incubation with antigen 1.8 μg./ml.) When blood from normal rabbits was incubated with the same concentration of the antigen no release took place. (In 3 rabbits the mean level of plasma histamine in untreated blood was 0.45 μg./ml. and after treatment 0.49 μg./ml.) In sensitized blood treated with antigen, sodium salicylate caused a graded reduction in the level of plasma histamine depending upon the concentration employed (Fig. 1).

In early experiments the control levels of plasma histamine were high, presumably because of a non-specific release, which also appeared to be reduced by the drug. Both sodium chloride and potassium chloride were effective inhibitors, but only at much higher molar concentrations than were needed with sodium salicylate. Since the regression lines for sodium chloride and potassium chloride were so close together it seemed likely that the inhibitory activity could be attributed to the anion. The inhibitory effect of sodium benzoate was found to equal that of sodium salicylate.

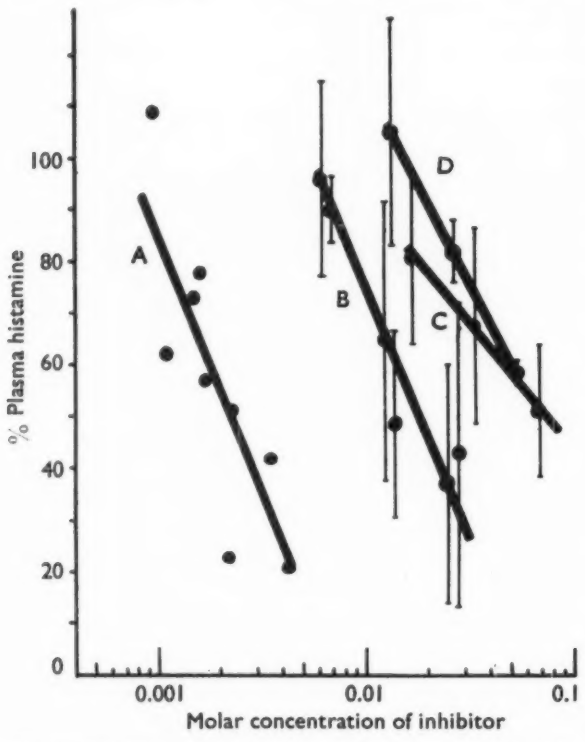

FIG. 1.—Inhibition of the *in vitro* anaphylactic release of histamine from rabbit blood cells. Blood from sensitized rabbits was incubated with the specific antigen (horse serum 1: 250) in the presence of inhibitors for 15 min. at 37.5° C. The plasma histamine level obtained in the presence of antigen + drug was compared with that found after treatment with antigen only (= 100%). A, 3-hydroxy-2-phenylcinchoninic acid; B, sodium salicylate and sodium benzoate; C, sodium chloride; D, potassium chloride. Standard deviations are shown by vertical bars.

Sodium salicylate, sodium chloride and sodium benzoate at the highest concentrations used in these experiments did not interfere with the extraction and assay of histamine (approx. 1 μg./ml.) from dilute plasma. Mean estimates of plasma histamine found in the presence of drugs expressed as percentages of that in untreated plasma were as follows: sodium salicylate 104% (4 expts.), sodium benzoate 98% (2 expts.), sodium chloride 102% (2 expts.).

Difficulties were experienced when testing 3-hydroxy-2-phenylcinchoninic acid (HPCA) for inhibitory activity since low recoveries of histamine were obtained when it was present in plasma. Attempts to remove the compound from plasma were unsuccessful, and its interfering effects were therefore neutralized by using "paired" samples of blood in the histamine release reaction. One sample contained HPCA (as the Na salt in 0.85% w/v NaCl, *p*H 7.1–7.4) during the incubation period when antigen was present, and the same amount of HPCA was added to the other sample only after further histamine release had been prevented in both samples by adding sodium oxalate 0.02 M. Of the compounds tested, the most effective in preventing histamine release was HPCA, which was about eight times as active as sodium salicylate. Even HPCA is, however, only active at high concentrations.

Confirmation of the inhibitory action of sodium salicylate on histamine release was obtained by determining the amount of histamine retained in the cells following *in vitro* anaphylactic reactions; the cellular fraction of blood showed in each case a marked fall in histamine concentration which corresponded to the increase found in the plasma. In the presence of sodium salicylate, this fall was either prevented or reduced (Table I), and in two samples of blood treated with varying concentrations of the salt graded reductions were obtained.

None of the inhibitors used caused an alteration of plasma *p*H as determined by indicator papers reading to 0.3 of a unit. However, in view of the possibility that changes of *p*H not detectable in this way could have been sufficient to interfere with histamine release, experiments were carried out to determine the action of varying amounts of hydrochloric acid on the release reaction. Inhibitory effects were only observed when the plasma *p*H fell from its normal value of 8.3 to 7.4.

TABLE I

INHIBITION OF THE *IN VITRO* ANAPHYLACTIC RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD BY SODIUM SALICYLATE

Blood from sensitized rabbits was incubated with the specific antigen (horse serum 1:250) for 15 min. at 37.5° C. Cellular or whole blood histamine was extracted by Code's method

Rabbit	Total Blood Histamine (μg./ml.)	Cellular Histamine (μg./ml. Blood)		Concentration of Sodium Salicylate (mg./ml.)	After Treatment with Antigen + Sodium Salicylate. Cellular Histamine (μg./ml. Blood)
		Control	After Treatment with Antigen		
7	7·0	5·7	2·8	4	6·1
9	1·9	1·4	1·4		
5	2·0	1·5	0·24	4	0·57
3	2·4	1·8	0·28	4	1·8
5		1·8	0·37	2	1·1
				4	1·5
3	1·7	1·3	0·55	1	0·50
				2	1·2
				4	1·6

In order to test the possibility that sodium salicylate might exert a permanent inhibitory action on the cells, dilute heparinized blood from a sensitized rabbit was incubated for 15 min. with sodium salicylate 4 mg./ml. The salicylate-containing plasma was then replaced by fresh plasma from the same sample of blood. The specific antigen was added and incubation was continued for a further 15 min. after which a sample of plasma was removed for testing. Knowing the histamine content of each plasma sample and the volume occupied by the cells, it was possible to calculate the total quantity of histamine liberated at each stage. When sodium salicylate was present only during the first incubation period, the quantity of histamine released during the second period (Table II) was comparable with that released by antigen in the absence of drug. Since very little histamine was released when antigen and sodium salicylate were present during both incubations this release must have taken place during the second incubation. It can be concluded, therefore, that the presence of sodium salicylate does not permanently interfere with the capacity of cells to release their histamine.

TABLE II

THE TRANSIENT INHIBITORY ACTION OF SODIUM SALICYLATE ON THE *IN VITRO* ANAPHYLACTIC RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD

Treatment	Histamine Released During Incubation Periods (μg./ml. Blood)		
	Rabbit 5 (Duplicates)		Rabbit 14
Blood incubated with specific antigen and sodium salicylate (4 mg./ml.) for two 15-min. periods	−0·05	−0·07	0·41
Blood incubated with specific antigen for two 15-min. periods	2·2	2·5	1·2
Blood incubated with sodium salicylate (4 mg./ml.) during the first 15 min. Salicylate-containing plasma was then replaced by fresh plasma plus the specific antigen and incubation continued for 15 min.	2·1	2·1	1·1*

* Antigen also present during the first incubation.

Histamine release in normal rabbit blood can be initiated by incubation with preformed washed antigen-antibody precipitate (P. B. Dews, personal communication), and experiments were carried out to determine whether sodium salicylate, dextran sulphate and heparin would inhibit this release. The antigen-antibody precipitate always caused a marked increase in the histamine concentrations in the plasma, and this was completely prevented by sodium salicylate 4 mg./ml. and by dextran sulphate I 2–4 mg./ml. and partially by heparin 2 mg./ml. (Table III). The histamine was not derived from the antigen-antibody precipitate itself, since incubation of the precipitate with rabbit plasma for 30 min. did not increase the concentrations of plasma histamine (the mean value in untreated plasma was 0.31 μg./ml. (5 determinations) and after incubation with the precipitate was 0.33 μg./ml.).

These facts suggested that the antigen-antibody precipitate was causing activation of the plasma, and this was tested experimentally. When normal blood was incubated with its own "activated"

TABLE III

THE *IN VITRO* RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD AND ITS INHIBITION BY SODIUM SALICYLATE, HEPARIN, AND DEXTRAN SULPHATE I

Normal dilute heparinized rabbit blood with or without inhibitor was incubated with washed antigen-antibody precipitate for 15 min. at 37.5° C. Histamine in the supernatant dilute plasma was extracted by McIntire's method

Expt.	Plasma Histamine (μg./ml. Blood)					
	Control	Blood Incubated with Antigen-Antibody Precipitate				
		Alone	+ Sodium Salicylate (4 mg./ml.)	+ Heparin* (2 mg./ml.)	+ Dextran Sulphate I (4 mg./ml.)	+ Dextran Sulphate I (2 mg./ml.)
1	0·27 0·21	1·5 1·9	0·30 0·33			
2	0·30 0·37	1·4 2·2	0·23 0·46			
3	0·27 0·32	1·9				
4	0·60	1·8		0·82		
5	0·20 0·15	1·2 1·1		0·70 0·57		0·17 0·17
6	0·37	1·7			0·29	

* 93 units/mg.

plasma (see Methods), there was almost always a marked increase in the histamine concentration of the plasma (Table IV). This release of histamine was inhibited by sodium salicylate 4 mg./ml., but heparin 2 mg./ml. did not appear to be effective.

This activation of rabbit plasma may be distinct from that which occurs during anaphylatoxin formation. Tests on isolated guinea-pig ileum showed that neither rabbit serum nor heparinized plasma

TABLE IV

THE *IN VITRO* RELEASE OF CELLULAR HISTAMINE IN RABBIT BLOOD BY "ACTIVATED" RABBIT PLASMA AND THE EFFECTS OF SODIUM SALICYLATE AND HEPARIN ON THE REACTION

"Activated" plasma was prepared by incubating rabbit plasma with antigen-antibody precipitate. At the end of the incubation period the precipitate was removed and the plasma incubated with dilute heparinized blood from the same rabbit

Experiment	Plasma Histamine μg./ml. of Blood				
	Blood Incubated with Untreated Plasma		Blood Incubated with "Activated" Plasma		
			Alone	+ Sodium Salicylate (4 mg./ml.)	+ Heparin (2 mg./ml.)
1	0·33		1·0 1·1	0·49 0·52	
2	0·56 0·87		1·9	0·56 0·64	
3	0·37		0·54	0·36	
4	0·60		0·64	0·19	
5		0·31 0·30	0·60 0·71		0·76 0·52
6		0·68	1·2		1·4
3		0·37	0·54		0·51
4		0·60	0·64		0·48
Mean percentage	58	67	100	44	96

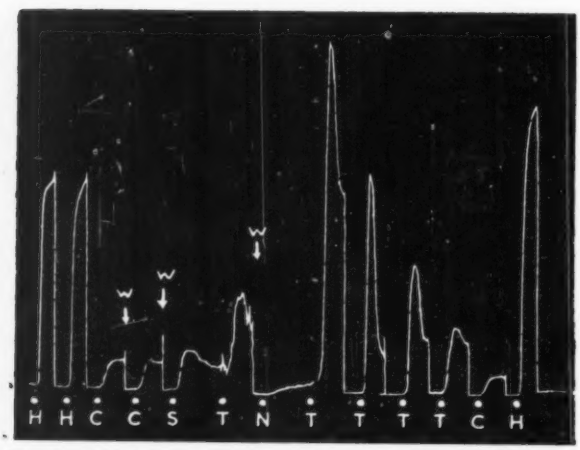

FIG. 2.—Inhibition of the action of rat serum agar anaphylatoxin on guinea-pig ileum by sodium salicylate. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine (as acid phosphate) 0.4 μg./ml.; C, control rat serum, 0.008 ml./ml.; W, organ-bath fluid replaced by fresh Tyrode solution; S, sodium salicylate added to bath fluid giving final concentration of 4 mg./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.008 ml./ml.; N, NaCl added to bath fluid giving final molarity equivalent to sodium salicylate 4 mg./ml.

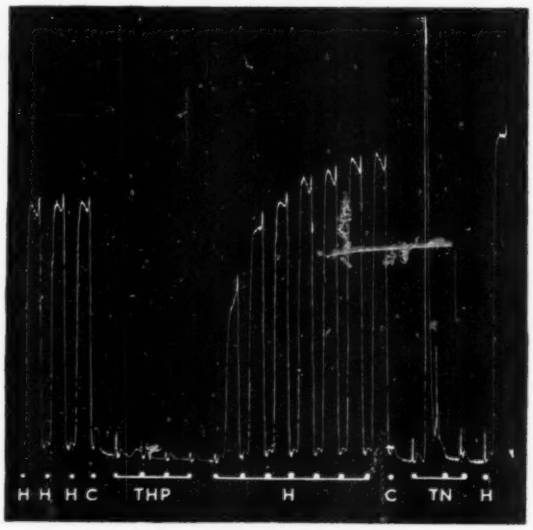

FIG. 3*a*

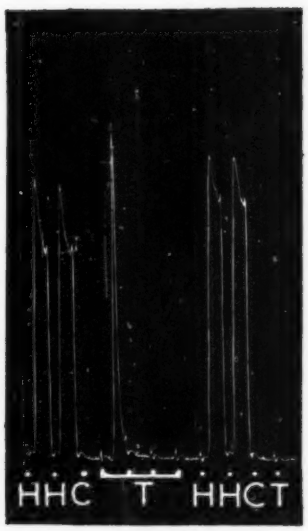

FIG. 3*b*

FIGS. 3*a* and 3*b*.—Inhibition of the action of rat serum agar anaphylatoxin by hydroxyphenylcinchoninic acid. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine, 0.03 μg./ml.; C, control rat serum, 0.004 ml./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml.; THP, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml. plus hydroxyphenylcinchoninic acid 0.5 mg./ml.; TN, rat serum after incubation with New Zealand agar (0.83 mg./ml.), 0.004 ml./ml. plus NaCl of equal molarity to hydroxyphenylcinchoninic acid 0.5 mg./ml.

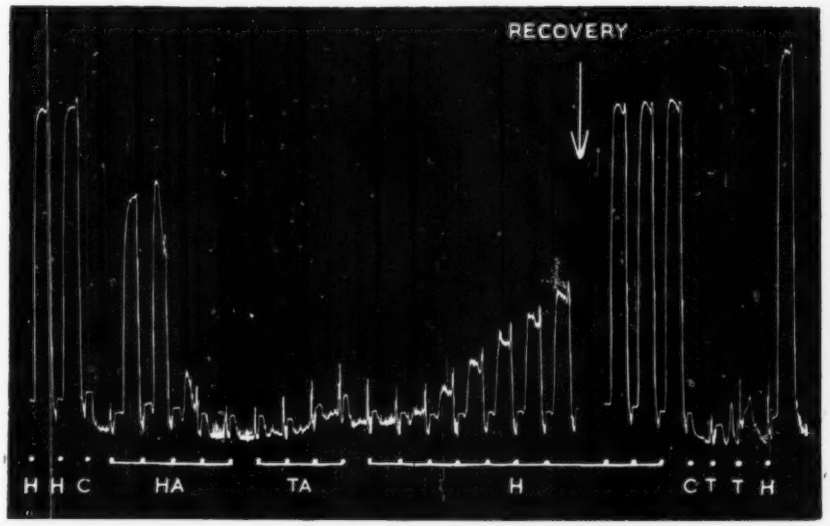

FIG. 4.—Inhibition of the effect of rat serum agar anaphylatoxin on guinea-pig ileum by mepyramine maleate. Isolated guinea-pig ileum suspended in Tyrode solution containing atropine sulphate 1 mg./l. at 36° C. H, histamine (as acid phosphate) 0.03 μg./ml.; C, control rat serum, 0.004 ml./ml.; HA, histamine (as acid phosphate) 0.03 μg./ml. plus mepyramine maleate 0.005 μg./ml.; TA, rat serum after incubation with New Zealand agar (0.83 mg./ml.) 0.004 ml./ml. plus mepyramine maleate, 0.005 μg./ml.; T, rat serum after incubation with New Zealand agar (0.83 mg./ml.) 0.004 ml./ml. *Recovery*, doses of histamine 0.03 μg./ml. until recovery of the gut had taken place.

(heparin 1.5 units/ml.) gave rise to an anaphylatoxin when incubated for 2 hr. with dextran sulphate D/3 0.1 mg./ml. or agar 0.83 mg./ml. although these concentrations were effective in producing anaphylatoxin when rat serum was used.

Both sodium salicylate and HPCA inhibited the action of rat serum agar anaphylatoxin on isolated guinea-pig ileum. Fig. 2 shows that sodium salicylate 4 mg./ml. prevented the action of anaphylatoxin without having any permanent inactivating effect on the gut; lower concentrations were not effective. The action of anaphylatoxin could be completely prevented by HPCA at a concentration of the order 0.5 mg./ml. but not without interfering with the response to histamine. Fig. 3a shows that in the presence of HPCA 0.5 mg./ml., 4 doses of anaphylatoxin failed to cause a contraction of guinea-pig ileum although, after recovering its sensitivity to histamine, the preparation responded to the same dose of anaphylatoxin. In a control experiment using a different strip of the same gut, complete desensitization was obtained by one dose of anaphylatoxin (Fig. 3b). This action of HPCA is in contrast to that of mepyramine maleate, which also prevented anaphylatoxin from producing a contraction but which rendered the gut insensitive to subsequent doses of anaphylatoxin after it had recovered its previous sensitivity to histamine (Fig. 4).

Sodium salicylate 4 mg./ml. and HPCA 1 mg./ml. did not prevent the development of an active anaphylatoxin when added to rat serum before its incubation with agar.

DISCUSSION

Available evidence suggests that the initial reactions leading to histamine release in rabbit blood occur in the plasma. Histamine release takes place when blood to which antibody has been added, or blood from a sensitized animal, is incubated with the specific antigen (Katz, 1940; Dragstedt, 1941; McIntire, Roth, and Sproull, 1950; Spain, Strauss, and Neumann, 1950), but the interaction between antigen and antibody need not occur in the presence of cells (McIntire *et al.*, 1950) although plasma is essential (Humphrey and Jaques, 1955). This suggests that release is initiated either by some action of the antigen-antibody complex upon the cells, or as a result of plasma activation, which could be brought about by the antigen-antibody complex itself, or by some reaction occurring during its formation.

The present results show that plasma which has been incubated with antigen-antibody precipitate acquires the ability to release a histamine-like substance in blood, and confirms that a similar release occurs when antigen-antibody precipitate is incubated with blood. However, any wettable surface may release histamine in rabbit blood (Code, 1952), and serum or plasma of various species is known to be activated by incubation with particulate matter (Wilson and Miles, 1946) so that the properties of preformed antigen-antibody precipitate do not necessarily represent those of the complex formed during *in vitro* anaphylactic reactions.

Since sodium salicylate inhibits histamine release either by antigen in blood from a sensitized animal, or by antigen-antibody precipitate or activated plasma in normal blood, it is unlikely that its action is due to interference with antibody or antigen-antibody complex (Coburn and Kapp, 1943). Further, the ability of cells to respond normally to antigen or anaphylatoxin after treatment with

concentrations of the drug capable of inhibiting histamine release makes it clear that damage to the cells is not preventing release.

Since the concentrations of sodium salicylate and HPCA which prevent histamine release in rabbit blood and from guinea-pig ileum are of the same order, a common site of action in the two preparations seems likely. The ability of both compounds to inhibit fibrinolysin (Ungar, Damgaard, and Hummel, 1952) could explain their activity against anaphylactic histamine release, since the enzyme is activated during antigen-antibody reactions in rabbit plasma (Geiger, 1952); but no evidence is available to suggest that it is activated in blood treated with preformed antigen-antibody precipitate or activated plasma. However, the failure of heparin to inhibit release by activated plasma, in contrast to its effectiveness against release due to antigen-antibody precipitate, is of interest, since heparin may prevent the activation of fibrinolysin although it is ineffective against the activated enzyme (Ungar and Mist, 1949).

Although activation of rabbit plasma bears a superficial resemblance to anaphylatoxin formation, failure to demonstrate activation of rabbit plasma or serum by agar and dextran sulphate D/3 under conditions in which rat serum was effective points to some fundamental difference between the two reactions. It seems unlikely that antigen-antibody precipitate incubated with blood forms an anaphylatoxin, since heparin, which partially prevents the resulting histamine release, does not prevent anaphylatoxin formation (Rocha e Silva, 1952). If protease activation is concerned in anaphylatoxin formation it should be possible to demonstrate it. Dextran sulphate D/3, dextran D, and agar, all release histamine in rabbit blood, and form anaphylatoxins when incubated with rat serum; but whereas dextran sulphate D/3 activates protease in serum from rats and rabbits, dextran D does not, and agar is ineffective in rat serum under comparable conditions (Haining, 1955, 1956).

The failure of sodium salicylate and HPCA to prevent anaphylatoxin formation is in contrast to the action of sodium citrate; this stops the activation of rat serum by agar and yet is ineffective against formed anaphylatoxin (Rocha e Silva, 1952). The mode of action of sodium salicylate and HPCA in preventing anaphylatoxin-induced contraction of the guinea-pig ileum is quite distinct from that of mepyramine maleate. Anaphylatoxins are known to release cellular histamine (Rocha e Silva, 1952). But in the presence of a specific histamine antagonist they fail to contract guinea-pig ileum, although histamine is released, as shown by the desensitization of the preparation to further doses. This can presumably be attributed to the blocking of histamine receptors by mepyramine. Sodium salicylate and HPCA must prevent the release of histamine since, in the presence of either drug, anaphylatoxin does not cause a contraction of smooth muscle and does not desensitize it. It seems likely that both drugs exert their inhibitory actions by an extracellular mechanism, since guinea-pig ileum which has been treated with anaphylatoxin, in the presence of sufficient of either compound to prevent a spasm, does not contract when the bath fluid is replaced by fresh Tyrode solution. It may be that in the presence of either drug the anaphylatoxin is not able to initiate the release mechanism because some factor in serum is inactivated or prevented from reaching a cell site. However, the possibility of these drugs having a transient intracellular action cannot be ruled out.

Summary

1. Factors influencing the *in vitro* release of histamine in rabbit blood are described.

2. Anaphylactic histamine release in rabbit blood is reduced by a lowering of plasma *p*H and by suitable concentrations of sodium chloride, potassium chloride, sodium benzoate, sodium salicylate, and 3-hydroxy-2-phenylcinchoninic acid. The most effective compound was 3-hydroxy-2-phenylcinchoninic acid, which is approximately eight times as active as sodium salicylate or sodium benzoate.

3. Sodium salicylate, heparin, and dextran sulphate of low molecular weight, inhibit, in normal blood, the histamine release due to incubation with washed antigen-antibody precipitate. Sodium salicylate is effective against release due to plasma activated by antigen-antibody precipitate.

4. Sodium salicylate and 3-hydroxy-2-phenylcinchoninic acid do not prevent the formation of anaphylatoxin but inhibit its action on isolated guinea-pig ileum. This property is shown not to depend upon an antagonism to histamine.

5. The significance of the results is discussed.

I thank Professor A. C. Frazer for his continued interest in this work; Dr. P. B. Marshall for helpful discussions; Dr. J. H. Humphrey, who suggested the modified formula for Freund antigen, and supplied the avian tubercle bacilli; Dr. C. R. Ricketts for dextran sulphate; and Herts Pharmaceuticals for a generous supply of 3-hydroxy-2-phenylcinchoninic acid.

This work was carried out during the tenure of a grant from the Colonial Products Research Council.

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Brit. J. Pharmacol. (1956), **11**, 364.

THE HYPERPNOEA PRODUCED BY INTRAVENOUS ADMINISTRATION OF SALICYLATES

BY

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(RECEIVED FEBRUARY 16, 1956)

An increase in pulmonary ventilation after administration of salicylate is well known and has been shown to be associated with a respiratory alkalosis (Rapoport and Guest, 1945; Boyle, Smull, and Wégria, 1947). Graham and Parker (1948) have suggested that this alkalosis is due to a primary action on respiration through stimulation of peripheral vagal nerve endings. This suggestion was based on their observation that the hyperpnoea produced by intravenous administration of salicylate to the anaesthetized cat and rabbit is abolished by bilateral vagotomy.

The development of a respiratory alkalosis after salicylate administration was confirmed by Reid, Watson, and Sproull, 1950; but its close association with a pronounced increase in protein katabolism, together with the later finding that salicylate is a powerful metabolic stimulant (Cochran, 1952, 1954) acting at the cellular level (Sproull, 1954), indicated that the primary action may be metabolic and not respiratory. For this reason, re-examination of Graham and Parker's claim to have localized the action at peripheral vagal nerve endings has been undertaken.

METHODS

Nine cats were used in the first series. These were anaesthetized with chloralose or with pentobarbitone sodium and then placed in an airtight box. The lungs were connected to the outside air by a tracheal cannula and tube. Intravenous injections were made from outside the box through a tube connected to a venous cannula. Changes in the volume of air inside the box were recorded by a tambour; these changes gave indirectly the volume of air respired. This method was found to be useful for qualitative measurements only.

More accurate and quantitative records of the ventilation were obtained by adaptation of the spirometer of Bernstein and Mendel (1953) to small animals. The arrangement is shown diagrammatically in Fig. 1. The water valves were later replaced by sensitive rubber flap valves. With this apparatus records were made from 9 decerebrate cats.

RESULTS

Qualitatively, the results obtained from anaesthetized animals were the same as those from decerebrate animals. A preliminary report has been published in which evidence is presented that anaesthesia does not affect the respiratory response resulting from increases in metabolism caused by injection of metabolic stimulants or by exercise (Ramsay, 1956). However, only the quantitative results from decerebrate cats are given here.

The effect of an intravenous injection of 0.05 g./kg. body weight sodium salicylate in a decerebrate

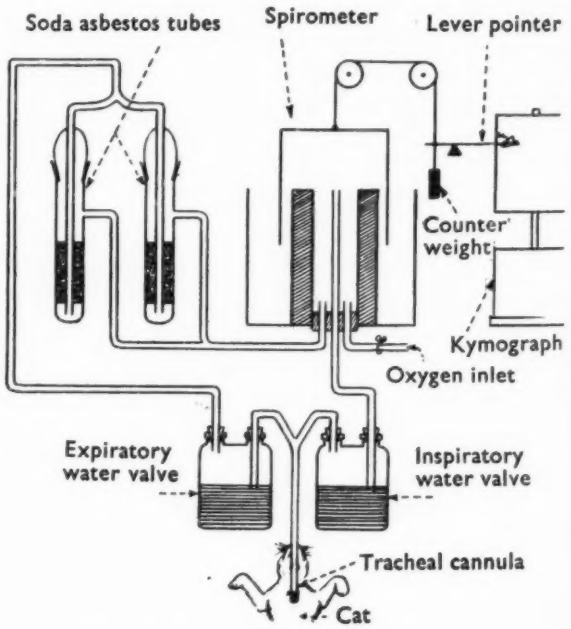


FIG. 1.—Schematic diagram of respirometer. The water valves were later replaced by sensitive rubber flap valves.

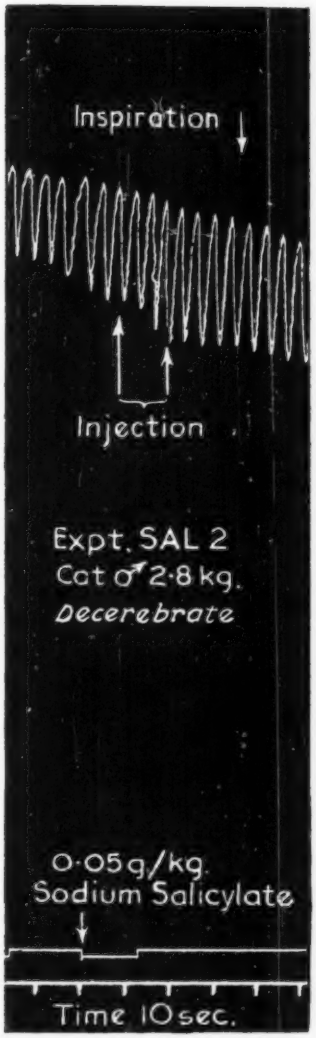


FIG. 2.—Cat, ♂, 2.8 kg., decerebrate. Respiratory response to 0.05 g./kg. body weight sodium salicylate intravenously. Inspiration, downstroke. Time, 10 sec.

cat is shown in Fig. 2. The pulmonary ventilation is increased from a resting level of 0.77 l./min. to 1.06 l./min. within a few seconds of the injection being made. (All values for ventilation are corrected to BTPS.)

After bilateral vagotomy the response is as seen in Fig. 3. The dose of sodium salicylate was 0.05 g./kg. body weight. This dose increased ventilation from 0.94 l./min. resting to 1.2 l./min. after injection.

An injection of 0.1 g./kg. body weight sodium salicylate produced up to a three-fold increase in ventilation in cats with intact vagi. After vagotomy, such a dose will also produce a stimulation of respiration. It was found, however, that vagotomized animals could not always maintain ventilation, respiratory movements becoming gasping and inadequate for survival.

In further experiments the effects on respiration of isomers of sodium salicylate were investigated. The absence of effect of doses of 0.1 g./kg. body weight of sodium *para*- and sodium *meta*-hydroxybenzoate when injected intravenously in decerebrate cats is shown in Fig. 4.

DISCUSSION

In the experiments on vagotomized animals by Graham and Parker (1948) it would seem that the slowing and deepening of respiration produced by vagotomy, combined with the insensitive stethographic recording method used, masked the respiratory response to salicylate administration.

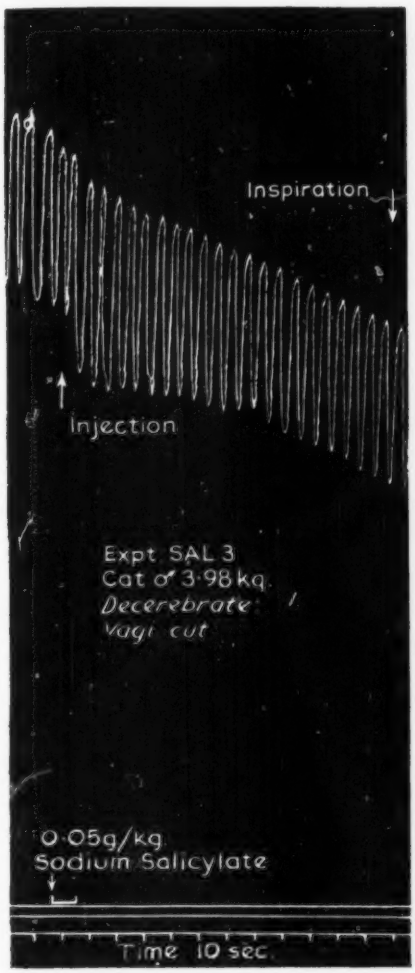
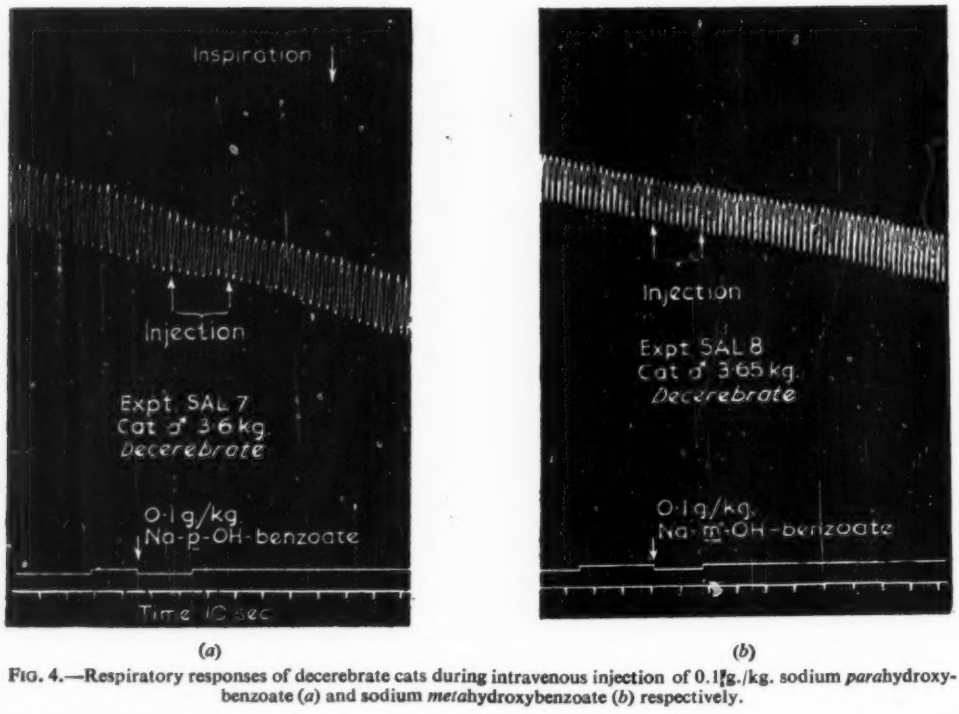


FIG. 3.—Respiratory response of a decerebrate, vagotomized cat to intravenous sodium salicylate (0.05 g./kg. body weight).



(*a*) (*b*)

FIG. 4.—Respiratory responses of decerebrate cats during intravenous injection of 0.1 g./kg. sodium *para*hydroxybenzoate (*a*) and sodium *meta*hydroxybenzoate (*b*) respectively.

The use of more accurate methods of recording respiration shows that vagotomy does not, in fact, abolish an immediate stimulatory effect on the respiration of the intravenous injection of sodium salicylate.

The site of stimulation cannot therefore be confined to receptors sending impulses along ingoing vagal fibres as suggested by Graham and Parker.

In vagotomized animals one of the control loops of the respiratory system has been severed. Thus the system is at a disadvantage when called upon to deal with the situation produced by a large dose of salicylate—such as 0.1 g./kg. as earlier described. It is considered that this is the reason for the frequent inability of the vagotomized animal to survive.

It would appear unlikely that, as Graham and Parker claim, intravenous administration of sodium salicylate "causes a primary stimulation of the mechanism of respiration."

There may be some significance in the finding that the isomers of sodium salicylate do not produce any stimulation of respiration. The isomers are known to be inert when administered to the human subject, and are ineffective in the relief of acute rheumatism (Stockman, 1920).

SUMMARY

1. Intravenous injection of sodium salicylate (0.05 g./kg. body weight) produces an immediate hyperpnoea in decerebrate and in anaesthetized cats.

2. The hyperpnoea produced by intravenous administration of sodium salicylate is seen after section of the vagi.

3. Intravenous injections of solutions of isomers of sodium salicylate do not produce a stimulation of breathing.

We wish to thank Professor R. C. Garry and Dr. James Reid for their help and encouragement. Part of the expenses were defrayed by the Rankin Medical Research Fund of the University of Glasgow.

We would also like to thank Miss Jane Wylie for invaluable technical assistance.

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THE ACTIVITY OF *p*-AMINOPHENOXYALKANE DERIVATIVES AGAINST *SCHISTOSOMA MANSONI*

BY

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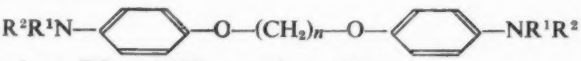
(RECEIVED MAY 12, 1956)

In recent publications, Raison and Standen (1954, 1955) have described the schistosomicidal activity of di-(*p*-aminophenoxy)alkane derivatives of formula



where R^1 and R^2 are H or alkyl.

The present communication is concerned with the schistosomicidal activity of related *p*-aminophenoxyalkane derivatives in which one of the *p*-aminophenyl groups is retained and the other is replaced by a differently substituted phenyl group or an alkyl group. These investigations have shown that schistosomicidal activity is found in many compounds of this general type and have provided further information on the relationship between structure and activity.

MATERIALS AND METHODS

All the compounds in this present series were tested against an Egyptian strain of *S. mansoni* passaged through *Australorbis glabratus*. The white mouse was used in all instances as host for the parasites. The mice were infected by the percutaneous route, each mouse being exposed to 130 cercariae (Standen, 1949, 1953). Determination of infection in mice was made by the identification of viable eggs in the faeces 56 days after exposure to the cercariae. At the time of treatment the infections were 63–70 days old.

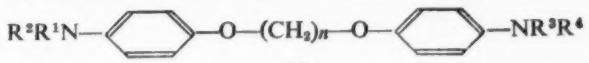
The drugs were given orally as aqueous solutions of their hydrochlorides (except for the non-basic compounds) twice daily for 5 days, and the mice were autopsied 7 days after completion of treatment. The hepatic portal system of each mouse was examined and the worms were removed from mesenteric, portal, and intrahepatic veins to determine numbers, sex, and proportional distribution in these organs. In addition to the indication of schistosomicidal activity provided by the degree of worm shift to the liver (Schubert, 1948; Standen, 1953), the final assessment of dose-response was made upon the proportion of dead worms found. Ten mice were used in each test, and the activity of a drug was estimated in terms of the proportion of dead worms found in relation to the total worms recovered in the group. Worms were considered dead only when completely ensheathed with inflammatory tissue and in process of disintegration.

The compounds mentioned in this paper were prepared by established chemical methods, and most of them have been described elsewhere (Wellcome Foundation, Ltd., 1954).

RESULTS

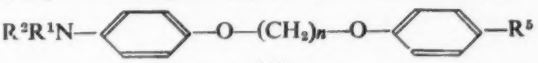
The compounds discussed in this paper may be divided conveniently into four types:

(i) Di-(*p*-aminophenoxy)alkane derivatives where the amino-groups are differently substituted (I; R^1, R^2, R^3, or R^4 may be H, Me or Et) (Table I).



(I)

(ii) *p*-Aminodiphenoxyalkane derivatives (II).

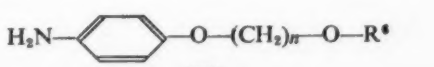


(II)

In this series, R^5 includes the following groups: H, CH_3, Cl, Br, OMe, OH, CH_2NH_2, NO_2, CN, $CONH_2$, CO_2Et, CO_2H, $COCH_3$, SO_2CH_3, $NH.COCH_3$, $NMe.COCH_3$, $NH.COC_4H_9$, $NHCO_2Et$, $NMe.CO_2Et$, $NH.CO_2C_5H_{11}$, $NHCONH_2$, and $NH.SO_2Me$, and the group $NR^1R^2 = NH_2$ (Table IIA), NHMe (Table IIB), and NMe_2 (Table IIC).

A few compounds of this type, but with one of the substituent groups in the *o*- or *m*-position, are listed in Table III.

(iii) *p*-Aminophenoxyalkoxyalkanes (III; $R^6 = CH_3$, $(CH_2)_2CH_3$, $(CH_2)_5CH_3$, *cyclo*hexyl) (Table IV).



(III)

(iv) Miscellaneous inactive compounds related to the above types (Table V).

The term " unit dose " in the Tables refers to the dose given orally twice daily for 5 days and is thus one-tenth of the total dose.

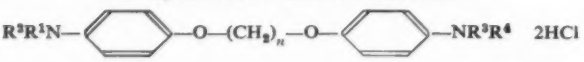
The Tables define the limits of activity for each compound in terms of worm kill. Since the relationship of hepatic shift to worm kill has previously been described (Raison and Standen, 1955), details of worm distribution are not included.

The compounds were well tolerated at the doses used. Toxicity studies on these and related compounds will form the subject of a separate report.

TABLE I

ACTIVITIES OF UNSYMMETRICAL DI-(*p*-AMINOPHENOXY)-ALKANE DERIVATIVES AGAINST *SCHISTOSOMA MANSONI*

For explanation of " unit dose " see Results



Compound No.	NR^1R^2	NR^3R^4	*n*	Unit Dose (mg./kg.)	Worms Killed %
241C53	NH_2	NHMe	5	100 50 25	100 78 36
242C53	NH_2	NHMe	6	50 25	100 83
524C54	NH_2	NHMe	7	50 25	100 93
158C55	NH_2	NMe_2	4	50 25	84 0
382C53	NH_2	NMe_2	5	50 25 12·5	99 90 7
383C53	NH_2	NMe_2	6	50 25 12·5	100 93 31
384C53	NH_2	NMe_2	7	25 12·5	98 42
385C53	NH_2	NMe_2	8	50 25 12·5	95 78 11
15C54	NHMe	NMe_2	6	50 25	100 53
49C54	NHMe	NMe_2	7	25	98
50C54	NHEt	NHMe	6	50 25	82 59

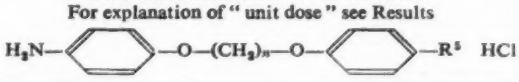
DISCUSSION

(i) *Di-(p-aminophenoxy)alkanes* (I).—In general, these compounds have the high activities which would be expected from their close resemblance to the symmetrical diamino-compounds (Raison and Standen, 1955). In the series where $NR^1R^2 = NH_2$ and $NR^3R^4 = NMe_2$, which was investigated over the range $n = 4$–8, the pattern of activity may

TABLE IIA

ACTIVITIES OF *p'*-SUBSTITUTED *p*-AMINODIPHENOXY-ALKANES AGAINST *S. MANSONI*

For explanation of " unit dose " see Results



Compound No.	R^5	*n*	Unit Dose (mg./kg.)	Worms Killed %
75C51	H	5	200 50	100 16
85C53	H	6	200 50	98 0
107C53	CH_3	5	200 50	51 0
108C53	CH_3	6	200 100 50	96 23 0
109C53	Cl	5	200 100	56 0
110C53	Cl	6	200 50	91 0
38C54	Br	6	200 50	96 0
111C53	OCH_3	5	200 100	64 0
112C53	OCH_3	6	200	0
181C53	OH	5	200	0
182C53	OH	6	200	0
269C53	CH_2NH_2	6	200 50	75 0
99C55	NO_2	3	200 50	78 2
325C54	NO_2	4	200 50	100 76
76C51	NO_2	5	200 50	87 0
86C53	NO_2	6	100 50	96 22
254C53	NO_2	7	200 50	97 0
267C53	NO_2	8	200	5
191C55	CN	3	200 50	100 7
326C54	CN	4	50 25 15	97 78 40
191C53	CN	5	200 50	62 0
192C53	CN	6	200 50 25	95 62 14
270C53	CN	7	200 50	81 0
284C53	CN	8	100 50	100 59
192C55	CN	9	200 50	96 8
303C53	$CONH_2$	5	200 50 25	100 50 0

TABLE IIA—*continued*

Compound No.	R^5	*n*	Unit Dose (mg./kg.)	Worms Killed %
304C53	$CONH_2$	6	200 50 25	80 36 5
136C53	CO_2Et	5	200 50	0 0
137C53	CO_2Et	6	200	2
138C53	CO_2H	5	200 50	36 1
139C53	CO_2H	6	200 50	52 0
119C54	$COCH_3$	6	200	0
13C54	SO_2CH_3	6	200 50	74 2
14C54	SO_2CH_3	7	200 50	94 72
102C55	$NH.COCH_3$	3	200 50	82 0
525C54	$NH.COCH_3$	4	200 50	94 5
349C53	$NH.COCH_3$	5	200 50	100 75
350C53	$NH.COCH_3$	6	200 50	100 19
526C54	$NH.COCH_3$	7	50 25	100 80
103C55	$NH.COCH_3$	8	200 50	97 53
648C54	$NH.COC_4H_9$	7	200 50	99 64
304C54	$NH.CO_2Et$	3	200 50	93 0
305C54	$NH.CO_2Et$	4	200 50	60 2
306C54	$NH.CO_2Et$	5	200 50 25 12·5	99 78 30 0
46C54	$NH.CO_2Et$	6	50 25 12·5 6·25	97 92 30 0
307C54	$NH.CO_2Et$	7	200 50 25	97 41 14
308C54	$NH.CO_2Et$	8	200 50	71 2
319C54	$NH.CO_2C_5H_{11}$	6	200 50	28 0
314C54	$NMe.CO_2Et$	3	200 50	67 0
245C54	$NMe.CO_2Et$	4	50 25 12·5	92 51 0
246C54	$NMe.CO_2Et$	5	50 25	80 13
143C54	$NMe.CO_2Et$	6	25 12·5 6·25	98 10 3

TABLE IIA—*continued*

Compound No.	R^5	*n*	Unit Dose (mg./kg.)	Worms Killed %
247C54	$NMe.CO_2Et$	7	50 25 12·5	98 56 3
248C54	$NMe.CO_2Et$	8	200 50 25	100 61 0
611C54	$NHCONH_2$	6	50 25	96 0
649C54	$NH.SO_2Me$	5	50	41

be compared with that of the symmetrical diamino- and bis-dimethylamino compounds. In the unsymmetrical compounds the graph of *n* against activity is similar to that for the symmetrical diamines, in that at 25 mg./kg. there is a rather broad spread of high activity ($n=5$–8 compared with $n=6$–9 for the symmetrical diamines), with peak activity at $n=7$ ($n=7$–8 for the symmetrical diamines), but there is no sign of the alternation in activity with *n* which is characteristic of the bis-dimethylamino-compounds.

(ii) p-*Aminodiphenoxyalkanes* (II).—In this series, some activity at 200 mg./kg. is found where NR^1R^2 is an amino-, methylamino- or dimethylamino-group and R^5 is any of a wide variety of different groups, including H. With $NR^1R^2=NH_2$, where $R^5=H$, CH_3, Cl, Br, OMe, OH, CH_2NH_2, CO_2Et, CO_2H, or $COCH_3$, activity was low with $n=5$ or 6, and further values of *n* were not studied. Similarly, low activity was found when $NR^1R^2=NMe_2$ and $R^5=H$, CH_3, Cl, or OMe and $n=5$ or 6. With $NR^1R^2=NH_2$, the following substituents in R^5 appeared to confer greater activity than those mentioned above, namely NO_2, CN, $CONH_2$, SO_2Me, $NH.COCH_3$, $NMe.COCH_3$, $NH.CO_2Et$, $NMe.CO_2Et$, $NHCONH_2$ and $NH.SO_2Me$. Several of these have been studied in corresponding methylamino- and dimethylamino- compounds, particularly in relation to changes in the value of *n*. Compounds where R^5 is $NH.COCH_3$, $NMe.COCH_3$, $NH.CO_2Et$ or $NMe.CO_2Et$ include some with very high activity.

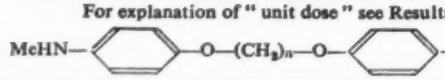
The changes in activity resulting from changes in either NR^1R^2, R^5 or *n* are not regular and predictable, except in a limited sense, but it is possible to make certain generalizations from those series which have been studied in sufficient detail, and to make some comparisons of the activity of these unsymmetrically substituted compounds with that of the previously studied symmetrical diamines.

Variation of Activity with n.—In the diamino-

TABLE IIB

ACTIVITIES OF *p'*-SUBSTITUTED *p*-METHYLAMINODIPHENOXYALKANES AGAINST *S. MANSONI*

For explanation of "unit dose" see Results



Compound No.	R^5	n	Unit Dose (mg./kg.)	Worms Killed %
285C53	H	6	200 50	100 29
100C55	NO_2	3	200 50	99 31
101C55	NO_2	4	200 50	100 33
217C53	NO_2	5	200 50	77 0
218C53	NO_2	6	200 50 25	100 75 19
255C53	NO_2	7	200 50	59 4
268C53	NO_2	8	200 50	55 54
193C55	CN	3	50 25	70 23
327C54	CN	4	25 12·5	98 18
336C53	CN	5	200 50	99 48
286C53	CN	6	100 50	91 21
287C53	CN	7	100 50	100 54
337C53	CN	8	200 50	97 22
645C54	$NH.COCH_3$	3	200 50	100 0
315C54	$NH.COCH_3$	4	25 12·5	97 16
316C54	$NH.COCH_3$	5	25 12·5	94 0
317C54	$NH.COCH_3$	6	25 12·5	100 22
318C54	$NH.COCH_3$	7	25 12·5	98 38
646C54	$NH.COCH_3$	8	50 25 12·5	98 87 7
329C54	$NMe.COCH_3$	4	25 12·5	95 6
209C55	$NMe.COCH_3$	7	50 25	100 80
168C54	$NH.CO_2Et$	4	200 50 25	100 58 3
552C54	$NH.CO_2Et$	5	50 25	100 83
553C54	$NH.CO_2Et$	6	25	95
554C54	$NH.CO_2Et$	7	50 25	86 27
169C54	$NMe.CO_2Et$	4	50 25 12·5	97 90 42
609C54	$NMe.CO_2Et$	5	50 25	99 44
144C54	$NMe.CO_2Et$	6	25 12·5	98 25
610C54	$NMe.CO_2Et$	7	25 12·5	95 12

diphenoxyalkanes studied by Raison and Standen (1955) it was observed that for a given series activity reached a peak for one or more values of n. A similar phenomenon, but with certain differences, is found in the present series, in which there are three types of activity/n relationship:

(a) *With one peak.* In the more active series showing this type of activity/n relationship, peak activity is usually at $n=6$ (e.g., II; $NR^1R^2=NH_2$ or NHMe; $R^5=NH.CO_2Et$) (Fig. 1, A), but may appear at $n=7$ (II; $NR^1R^2=NMe_2$; $R^5=NH.COCH_3$). In the symmetrical diamino-compounds showing this pattern of activity, peak activity is at $n=7$ or 8.

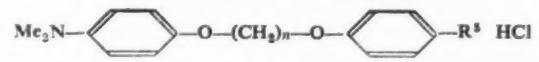
(b) *With two peaks.* Some of the most active series of compounds show a type of response providing one main peak and a second subsidiary one. Good examples are the series (II; $NR^1R^2=NH_2$; $R^5=NMe.CO_2Et$) (Fig. 1, B) with the main peak at $n=6$ and a smaller peak at $n=4$, and (II; $NR^1R^2=NHMe$; $R^5=NH.COCH_3$) in which the main peak is at $n=7$ and the minor one again at $n=4$. In some series, however, the peak at $n=4$ has become the major one, e.g., in (II; $NR^1R^2=NHMe$; $R^5=NMe.CO_2Et$), where the peak at $n=4$ is slightly higher than that at $n=6$, and in (II; $NR^1R^2=NHMe$; $R^5=CN$) where the peak at $n=4$ is very high in relation to the general level of activity of the series, and in comparison with the subsidiary peak at $n=7$. This pattern of activity is strikingly similar to that found in some of the symmetrical bis-alkylamino-compounds, in which the second peak is usually at $n=4$ and may be the higher peak or subsidiary to the main peak at $n=7$–8, according to variation in alkyl substitution.

(c) *Alternation.* This phenomenon, with activity alternately rising and falling for successive values of n, appears in two series (II; $NR^1R^2=NH_2$; $R^5=CN$) (Fig. 1, C) and, less clearly, (II; $NR^1R^2=NHMe$; $R^5=NO_2$). In the former, activity is higher for even values of n (4, 6, 8) and

TABLE IIC

ACTIVITIES OF *p'*-SUBSTITUTED *p*-DIMETHYLAMINO-DIPHENOXYALKANES AGAINST *S. MANSONI*

For explanation of " unit dose " see Results

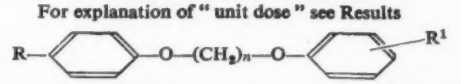


Compound No.	R^5	*n*	Unit Dose (mg./kg.)	Worms Killed %
91C53	H	5	200 50	100 5
92C53	H	6	200 50	62 0
130C53	CH_3	5	200 100	60 36
131C53	CH_3	6	200 100	82 16
132C53	Cl	5	200 100	88 35
133C53	Cl	6	200	7
134C53	OCH_3	5	200 50	96 0
135C53	OCH_3	6	200	0
157C55	NO_2	4	200 50	29 0
93C53	NO_2	5	100 50	44 0
94C53	NO_2	6	100	25
351C53	NO_2	7	200 50	61 9
352C53	NO_2	8	200 50	0 0
334C53	CN	5	200 50	81 26
335C53	CN	6	200 50 25	73 53 8
405C54	$NH.COCH_3$	4	50 25	96 6
406C54	$NH.COCH_3$	5	50 25	99 44
407C54	$NH.COCH_3$	6	50 25	100 45
408C54	$NH.COCH_3$	7	25 12·5	100 26
647C54	$NH.COCH_3$	8	50 25	92 0
47C54	$NMe.COCH_3$	6	50 25	100 55
235C55	$NMe.COCH_3$	7	25	99
527C54	$NH.CO_2Et$	4	200 50 25	87 75 4
528C54	$NH.CO_2Et$	5	50 25	100 24
529C54	$NH.CO_2Et$	6	50 25 12·5	94 64 18
530C54	$NH.CO_2Et$	7	200 50 25	100 84 65
555C54	$NMe.CO_2Et$	4	200 50 25	100 90 15
556C54	$NMe.CO_2Et$	6	50 25 12·5	98 83 84

TABLE III

ACTIVITIES OF SUBSTITUTED AMINODIPHENOXY-ALKANES AGAINST *S. MANSONI*

For explanation of " unit dose " see Results



Compound No.	R	R^1	*n*	Unit Dose (mg./kg.)	Worms Killed %
612C54	NH_2	*m*-NH_2	6	200 50	97 0
613C54	NH_2	*m*-$NH.CO_2Et$	6	200 50	99 32
614C54	$NH.CO_2Et$	*m*-NH_2	6	200 50	6 0
615C54	$NH.COCH_3$	*m*-NH_2	6	200 50	24 0
650C54	NH_2	*o*-$NH.CO_2Et$	7	200 50	36 0

lower for odd values of *n* (3, 5, 7, 9), and at low doses there is a marked peak at $n=4$. The only symmetrical type showing this behaviour is the bis-dimethylamino-series, but here the odd values of *n* give higher activity than even values, with peak activity at $n=7$.

Variation of Activity with NR^1R^2.—In general, for a single group R^5, the level of peak activity is not very different whether NR^1R^2 is NH_2, NHMe or NMe_2, although there may be marked differences in the shapes of the graphs of activity against *n*. For example, in the acetamido-series (II ; $R^5 = NH.COCH_3$), at 25 mg./kg., the methylamino-compounds have very high activity over the whole range $n=3$ to $n=8$, whereas the amino- and dimethylamino-compounds have peaks at $n=7$, but at 12.5 mg./kg. peak activity at $n=7$ is very similar in the amino-, methylamino- and dimethylamino-series.

Variation of Activity with R^5.—The fact that the group R^5 in compounds of type (II) is capable of such wide variation without loss of schistosomicidal

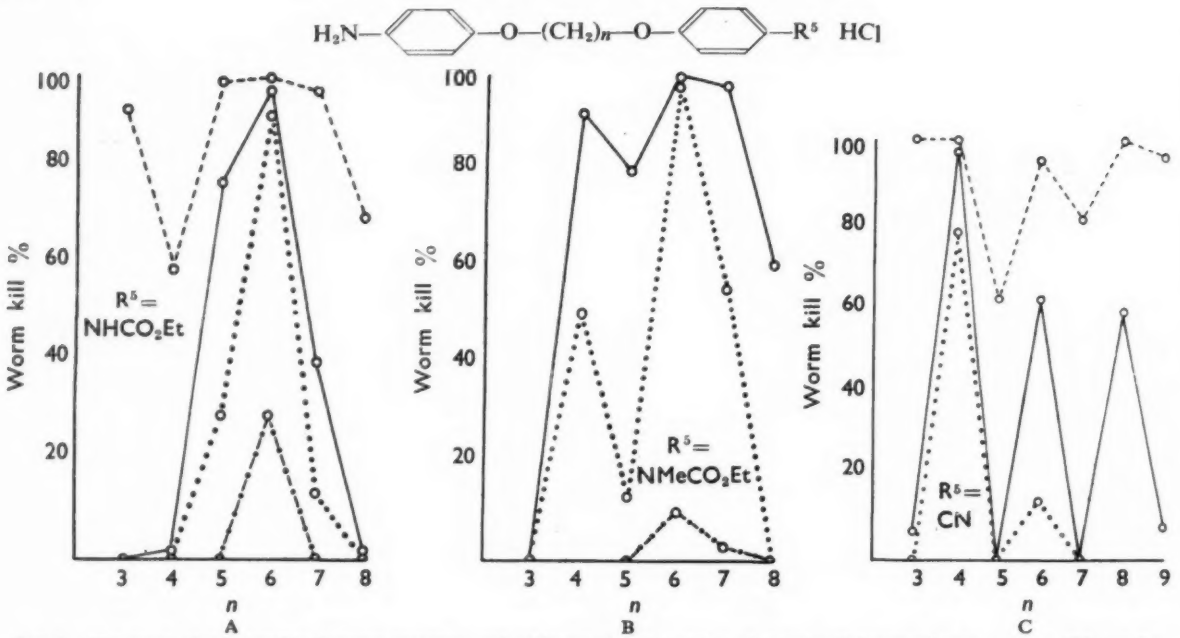


FIG. 1.—The types of relationship between structure and schistosomicidal activity of *p*-aminodiphenoxyalkanes. - - - - 200 mg./kg. ——— 50 mg./kg. .... 25 mg./kg. ·–·–·– 12.5 mg./kg.

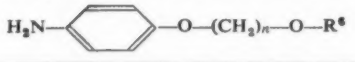
activity indicates clearly that only one *p*-amino-group in the diphenoxyalkane structure is essential for schistosomicidal activity. However, the nature of R^5 has a powerful influence on the degree of activity, which is completely lost when R^5=OH (as in 181C53 and 182C53, Table IIA), but is very high when, for instance, R^5=NH.CO_2Et (as in 46C54, Table IIA). The generally high activity of compounds where R^5=NH.$COCH_3$, NMe.$COCH_3$, NH.CO_2Et and NMe.CO_2Et is striking, since these groups are all capable of hydrolysis to amino-groups, and it is interesting to consider whether the activity of these compounds is attributable to the formation of the active diamino-compounds by hydrolysis in the body. High activity is also encountered, however, when R^5 is not a potential amino-group, as in the compound 326C54 (Table IIA), where R^5=CN.

The di-(*p*-acetamidophenoxy)alkanes and di-(*p*-ethoxycarbonamidophenoxy)alkanes are inactive, but this could be due to the prevention of their absorption and hydrolysis in the body by their unsuitable physical properties—such as lack of water solubility. The bis-(*m*-aminophenoxy)-alkanes are also inactive (Raison and Standen, 1955), but the presence of one *m*- and one *p*-aminophenoxy group (as in 612C54, Table III) gives moderate activity similar to that found in the monoamino-compound 85C53 (Table IIA), as might be expected if only the *p*-aminophenoxy group conferred the activity. The compound 614C54 (Table III), with a *m*-amino and a *p'*-

TABLE IV

ACTIVITIES OF *p*-AMINOPHENOXYALKOXYALKANES AGAINST *S. MANSONI*

For explanation of " unit dose " see Results

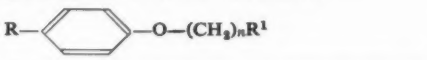


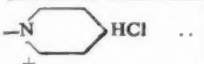
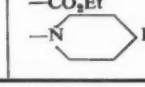
Compound No.	R^6	*n*	Unit Dose (mg./kg.)	Worms Killed %
328C54	CH_3	4	200 50	92 0
35C55	CH_3	5	200 50	83 0
369C53	CH_3	6	200 50	98 8
11C54	CH_3	7	200 50 25	97 82 0
121C54	CH_3	8	100 50 25	100 69 0
182C54	$(CH_2)_2CH_3$	6	100 50 25	98 22 4
183C54	$(CH_2)_2CH_3$	7	100 50 25	98 34 2
313C53	$(CH_2)_5CH_3$	5	200 50	98 0
314C53	$(CH_2)_5CH_3$	6	200 50	77 0
301C53	*cyclo*hexyl	5	200 50 25	92 91 12
302C53	*cyclo*hexyl	6	100 50	100 21

TABLE V

PHENOXYALKANE DERIVATIVES INACTIVE AGAINST *S. MANSONI* AT A UNIT DOSE OF 200 MG./KG.

For explanation of " unit dose " see Results



R	R^1	*n*
	$-O-C_6H_5$	6
$-\overset{+}{N}Me_3\ I^-$	$-O-C_6H_5$	5; 6
$-\overset{+}{N}Me_3\ I^-$	$-O-C_6H_4-NO_2(p)$	5; 6
$-NO_2$	$-O-C_6H_5$	6
$-NH.COCH_3$	$-O-C_6H_4-NO_2(p)$	6
$-H$	$-O-(CH_2)_5.CH_3$	6
$-CH_2.NH_2\ HCl$	$-OMe$	6
$-NH_2\ HCl$	$-OH$	6; 7
$-NH_2\ HCl$	$-O.COCH_3$	6
$-NH_2\ HCl$	$-H$	3; 4; 6; 12
$-NH_2\ HCl$	$-CO_2H$	5; 6
$-NH_2\ HCl$	$-CO_2Et$	5; 6
$-NH_2\ HCl$		5; 6

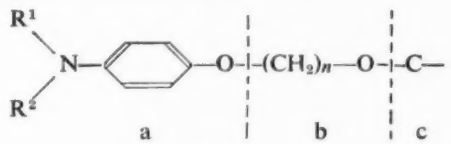
ethoxycarbonamido group, has physical properties similar to those of the active *p*-amino-*p'*-ethoxycarbonamido compound 46C54 (Table IIA), but it is very much less active than the *m*-*p'*-diamino compound, 612C54, which would result from hydrolysis of the ethoxycarbonamido group. Similarly, the *m*-amino-*p'*-acetamido-compound 615C54 (Table III) is much less active than the *m*-*p'*-diamine. These results suggest that the high activity of compounds where $R^5 = NH.COCH_3$ or $NH.CO_2Et$ is not accounted for simply on the basis of their hydrolysis *in vivo* to the diamino-compounds.

(iii) p-*Aminophenoxyalkoxyalkanes* (III).—The general level of activity in this series, as far as it has been studied, is only moderate, but shows features already observed in other types. When $R^6 = CH_3$ and *n* is varied, activity rises sharply to a peak at $n=7$ and decreases again at $n=8$. For a constant value of $n=6$, there is little change in activity whether $R^6 = CH_3$, $(CH_2)_2CH_3$, $(CH_2)_5CH_3$, or *cyclo*hexyl.

The most interesting aspect of this series of compounds is the demonstration that for schistosomicidal activity only one ring is necessary, and this provides a further simplification of the structure required to exhibit schistosomicidal activity.

Structural Requirements for Schistosomicidal Activity.—The foregoing investigations have shown that schistosomicidal activity is found in a very large range of compounds, some of which represent considerable modifications of the original diaminodiphenoxyalkane structure. On the basis of the present results, and those of Raison and Standen (1955), it is possible to attempt a definition of the minimum structural requirements for activity to appear. Such a definition will serve the dual purpose of summarizing the large amount of information already obtained, and of indicating other points requiring investigation. It will clearly be subject to modification in the light of further work which is proceeding and will be described in future communications.

The active compounds so far described all have the partial structure (IV) in common, and the inactivity of the compounds listed in Table V suggests that all the features present in this structure must be combined to produce a schistosomicidal compound. The variations which are permissible in the different portions of the basic structure (IV) may be summarized as follows:



(IV)

(a) The *p*-aminophenoxy-group may be varied with respect to the H or alkyl groups R^1 and R^2, but activity soon diminishes with increasing size of these alkyl groups (Raison and Standen, 1955).

(b) The value of *n* in the central carbon chain may be varied over the range about 3–9, but the values most often found in the most active compounds are 4 and 6–8.

(c) The carbon atom may be part of a wide variety of alkyl or substituted phenyl groups, certain of the latter giving highest activity.

In the light of these conclusions, it is interesting to speculate further on the possibility, suggested by Raison and Standen (1955), that the observed schistosomicidal activity of these compounds might represent a summation of intrinsic schistosomicidal activity and some property which determines the behaviour of the compounds in the host and the parasite. It seems reasonable to suppose that the intrinsic activity might reside in the portion (a) of (IV), since this appears to be the part of the molecule susceptible to least alteration without loss of activity. The parts (b) and (c) of (IV) might then be thought to be responsible for facilitating the attack on the parasite by their influence on absorption and metabolism in the host and parasite. To follow the idea further, it seems possible that the active compounds could all be converted in the body into *p*-aminophenol or a simple derivative by hydrolysis of the ether linkages and

conversion of the group $-NR^1R^2$ into NH_2, for it is known that fission of alkyl aryl ethers (Williams, 1947a) and demethylation of aromatic dimethylamino-groups (Williams, 1947b) may occur in the animal.

p-Aminophenol itself, however, has shown no schistosomicidal activity at non-toxic doses, whether administered orally or parenterally. The same is true of some other possible metabolites which could arise by partial breakdown of the structure (IV)—for example, the alcohols and carboxylic acids in Table V. It may be, of course, that such compounds do not reach the appropriate site of action in the parasite because of their lack of the structures which it is suggested are concerned with absorption. It is clear that such questions can be settled only by investigations into the mode of action of these compounds and their fate in the animal, and work on these lines is in progress.

Summary

1. Diphenoxyalkanes with different primary, secondary or tertiary amino-groups in the *p*-positions have schistosomicidal activity similar to that of the symmetrical *p* : *p'*-diaminodiphenoxyalkanes.

2. Diphenoxyalkanes, having a *p*-amino group in only one ring, and any of a variety of substituents in the other ring, also have schistosomicidal activity, although this is in general lower than that of the diaminodiphenoxyalkanes. However, where the other group is acetamido-, acetmethylamido-, ethoxycarbonamido-, ethoxycarbonmethylamido-, or cyano-, high activity appears.

3. *p*-Aminophenoxyalkoxyalkanes have moderate schistosomicidal activity.

4. An attempt is made to define the minimum structural features necessary for activity in this series, and the relationship between structure and activity is briefly discussed.

5. *p*-Aminophenol, and a few of its derivatives which are possible metabolites of these compounds, have shown no schistosomicidal activity.

We are indebted to Dr. C. G. Raison, of the Wellcome Research Laboratories, for the provision of some of the compounds mentioned in this paper. We are pleased to acknowledge the valuable technical assistance of Mr. W. E. King with the chemical work, and Mrs. Rosemary Richards and Mr. K. A. Fuller with the biological work.

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EFFECT OF RING-SUBSTITUENTS ON THE ACTIVITY OF DIPHENOXYALKANES AGAINST *SCHISTOSOMA MANSONI*

BY

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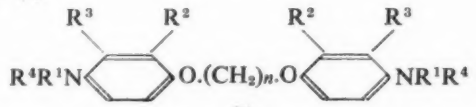
The discovery by Raison and Standen (1955) of the schistosomicidal activity of symmetrical diaminodiphenoxyalkanes led us to examine similar compounds in which the benzene rings have been further substituted as depicted in the general formula (I)



(I)

where alternatively R^2 or $R^3 = CH_3$, OCH_3, Cl, OH, NH_2 ; n, R^1 and R^4 have also been subjected to considerable variation. A few compounds with two methyl substituents in each ring and some analogous naphthalene compounds have also been studied.

The literature does not offer clear guidance on what might be expected from such modifications. According to Northey (1948) uniformly low activities have been encountered in derivatives of the sulphonamides in which the benzene nucleus has been substituted, and various compounds with alkyl, halogen, hydroxyl and nitro- substituents have been reported as inactive ; but Quan, Daniels, and Meyer (1954) have found that three derivatives of sulphathiazole of this type with methyl or chloro-substituents are at least as active against bacteria as sulphathiazole itself, though they are somewhat more toxic. The introduction of alkyl groups into an N^4-heterocyclic nucleus of such sulphanilamide derivatives as sulphadiazine modifies, but never destroys, the activity. Recently the effect of substitution on the antitubercular activity of *iso*nicotinic hydrazide has been investigated in detail (Isler, Gutman, Straub, Fust, Bohni, and Studer, 1955) ; all compounds with functional substituents are practically inactive, and alkyl substituents markedly reduce activity except with 2-methyl*iso*nicotinic hydrazide, which has similar activity to the unsubstituted compound. With the diamidines, a series formally analogous to ours, introduction of a hydroxyl group into stilbamidine has afforded hydroxystilbamidine, a versatile drug, free of the toxicity that has precluded the therapeutic use of stilbamidine itself ; with other drugs of this series, substituents have modified, without usually destroying, activity (Wien, 1946). The chemical effect of a substituent may be to block the metabolic course followed by the unsubstituted compound, or itself to serve as a point of metabolic attack—as has been postulated by Walker (1947) for the methyl group of inactive quinaldine analogues of certain quinoline antimalarial drugs —or to activate or deactivate positions of the benzene ring with consequential effects on metabolism. It is probably for the last-named reason that the 6-methoxyl group of the 8-aminoquinoline antimalarial drugs is essential to activity, facilitating their conversion into 5:6-quinones (Drake and Pratt, 1951 ; Taylor, Josephson, Greenberg, and Coatney, 1952).

In our series, introduction of any of the groups named above has always resulted in a big fall of activity, more often than not in complete loss, at our chosen maximum dose of 200 mg./kg. The effect on toxicity has not been studied in detail, but there were clear indications that several of the substituted compounds, particularly those of greater chain-length, are more acutely toxic than the unsubstituted analogues.

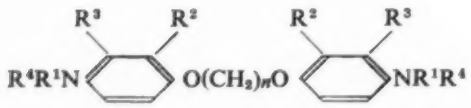
MATERIALS AND METHODS

The compounds described in this paper were given orally to mice infected with an Egyptian strain of *Schistosoma mansoni*. Treatment was started 63 days after the animals had been exposed to infection, and dosage was carried out twice daily for 5 consecutive days. Autopsy was made 7 days after completion of treatment and the result assessed as described by Raison and Standen (1955).

RESULTS

Methyl Substituents.—Whereas the primary amines (I ; $R^1 = R^4 = H$) of the unsubstituted

TABLE I



Compound Number	*n*	R^1	R^4	R^2	R^3	Unit Dose (mg./kg.)	Worms Killed %
A. Methyl Substituents : Primary							
170C53	3	H	H	H	CH_3	200	0
171C53	4	H	H	H	CH_3	200	Toxic
186C53	5	H	H	H	CH_3	200	64
						50	0
187C53	6	H	H	H	CH_3	200	67
						50	0
	7–10	H	H	H	CH_3	200	0
142C53	3	H	H	CH_3	H	200	60
						50	0
117C53	4	H	H	CH_3	H	200	79
						50	1
143C53	5	H	H	CH_3	H	200	2
144C53	6	H	H	CH_3	H	200	0
	7–10	H	H	CH_3	H	50	Toxic
B. Methyl Substituents : Secondary							
28C53	3	CH_3	H	CH_3	H	200	67
						50	0
29C53	4	CH_3	H	CH_3	H	200	68
						50	0
30C53	5	CH_3	H	CH_3	H	200	94
						50	8
31C53	6	CH_3	H	CH_3	H	200	94
						50	4
141C53	7	CH_3	H	CH_3	H	200	99
						50	28
75C53	8	CH_3	H	CH_3	H	200	78
						50	4
	9–10	CH_3	H	CH_3	H	200	Toxic
69C53	3	C_2H_5	H	CH_3	H	200	100
						50	0
70C53	4	C_2H_5	H	CH_3	H	200	99
						50	35
						25	8
101C53	5	C_2H_5	H	CH_3	H	200	68
						50	0
77C53	6	C_2H_5	H	CH_3	H	200	100
						50	3
78C53	8	C_2H_5	H	CH_3	H	200	96
						50	0
79C53	10	C_2H_5	H	CH_3	H	200	Toxic
C. Methyl Substituents : Tertiary							
	3, 5–8	CH_3	CH_3	H	CH_3	200	0
225C53	3	CH_3	CH_3	CH_3	H	200	45
103C53	4	CH_3	CH_3	CH_3	H	200	70
						50	0
290C53	5	CH_3	CH_3	CH_3	H	200	52
116C53	6	CH_3	CH_3	CH_3	H	200	95
494C55	7	CH_3	CH_3	CH_3	H	200	100
						50	3
169C53	8	CH_3	CH_3	CH_3	H	200	73
						50	0
	9–10	CH_3	CH_3	CH_3	H	200	Toxic
D. Methoxyl Substituents : Primary							
	3, 4, 6	H	H	H	OCH_3	200	0
	3, 4	H	H	OCH_3	H	200	0
72C53	5	H	H	OCH_3	H	200	12
73C53	6	H	H	OCH_3	H	200	5
102C53	7	H	H	OCH_3	H	200	94
						50	5
97C53	8	H	H	OCH_3	H	200	92
						50	6
223C53	9	H	H	OCH_3	H	200	80
						50	0
	10, 11	H	H	OCH_3	H	200	0

TABLE I—*continued*

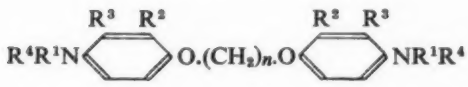
Compound Number	*n*	R^1	R^4	R^2	R^3	Unit Dose (mg./kg.)	Worms Killed %
E. Methoxyl Substituents : Secondary							
169C55	3	C_2H_5	H	OCH_3	H	200	0
170C55	4	C_2H_5	H	OCH_3	H	200	6
171C55	5	C_2H_5	H	OCH_3	H	200	24
172C55	6	C_2H_5	H	OCH_3	H	200	67
						50	0
596C55	7	C_2H_5	H	OCH_3	H	200	75
						50	0
173C55	8	C_2H_5	H	OCH_3	H	200	98
						50	5
635C55	9	C_2H_5	H	OCH_3	H	200	100
						50	34
F. Methoxyl Substituents : Tertiary							
	3–6	CH_3	CH_3	OCH_3	H	200	0
319C53	7	CH_3	CH_3	OCH_3	H	200	100
257C53	8	CH_3	CH_3	OCH_3	H	200	52
						50	0
60C55	9	CH_3	CH_3	OCH_3	H	200	93
						50	0
224C53	10	CH_3	CH_3	OCH_3	H	200	8
G. Other Substituents : Primary							
367C53	6	H	H	H	Cl	200	0
	3–7	H	H	Cl	H	200	0
370C53	8	H	H	Cl	H	200	24
	9–10	H	H	Cl	H	200	0
	6, 7	H	H	H	OH	200	0
	5, 7	H	H	OH	H	200	0

series (Raison and Standen, 1955) give 100% activity at 200 mg./kg. for $n=3$ to 9 and at 25 mg./kg. for $n=6$ to 8, none of the corresponding compounds in which R^2 or $R^3=CH_3$ is 100% active at 200 mg./kg. for $n=3$ to 10, only a few showing any activity at all (Table I*A*).

The secondary amines of the ($R^2=CH_3$, $R=H$) series (Table I*B*) are markedly more active than the primary amines, a difference not shown in other series studied here, but their activity at 200 mg./kg. is hardly superior to that of the unsubstituted compounds at 25 mg./kg. although a certain parallelism can be observed (Fig. 1). Full comparison is not possible owing to the high toxicity of the compounds of greater chain-length. As with the unsubstituted series the methylamino- and ethylamino-compounds behave very similarly (Fig. 2).

The tertiary amines of the (I ; $R^2=H$, $R^3=CH_3$) series (Table I*C*) are inactive for the examples tested, whereas those of the (I ; $R^2=CH_3$, $R^3=H$) series show some activity for $n=3$ to 8, but greatly reduced compared with the unsubstituted series and without the marked alternation of properties characteristic of the latter.

Methoxyl Substituents.—The primary amines of the series (I ; $R^2=H$, $R^3=OCH_3$) are inactive at the maximum dosage for the examples tested ($n=3$, 4, 6) (Table I*D*), but those of the series (I ; $R^2=OCH_3$, $R^3=H$) show full activity at 200 mg./kg. for $n=7$ to 9 with almost complete loss of



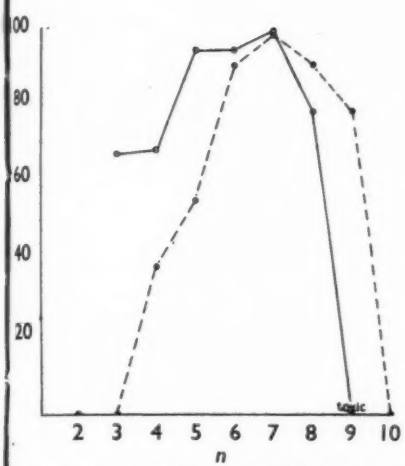


FIG. 1:——$R^1=R^2=CH_3$; $R^3=R^4=H$, at 200 mg./kg. - - - - $R^1=CH_3$; $R^2=R^3=R^4=H$, at 25 mg./kg.

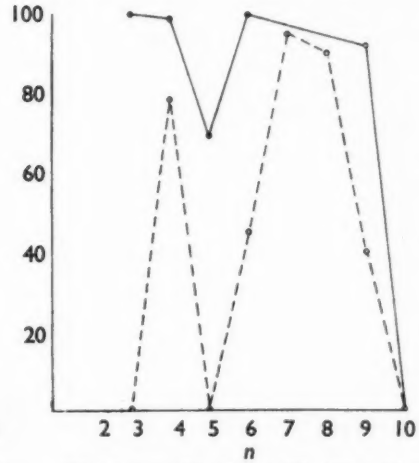


FIG. 2:——$R^1=C_2H_5$; $R^2=CH_3$; $R^3=R^4=H$, at 200 mg./kg. - - - - $R^1=C_2H_5$; $R^2=R^3=R^4=H$, at 25 mg./kg.

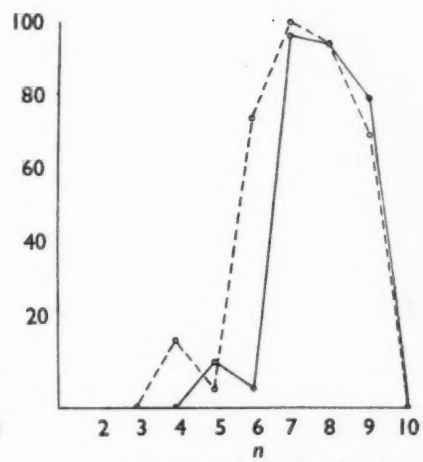


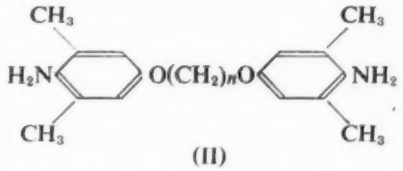
FIG. 3:——$R^2=OCH_3$; $R^1=R^3=R^4=H$, at 200 mg./kg. - - - - $R^1=R^3=R^4=H$, at 25 mg./kg.

FIGS. 1, 2, and 3.—The effect of further substitution (methyl in Figs. 1 and 2; methoxyl in Fig. 3) in the benzene rings on the activity of di-*p*-methylamino- (in Fig. 1); di-*p*-ethylamino- (in Fig. 2); and di-*p*-amino- (in Fig. 3) diphenoxyalkanes of chain-length $n=3$–10, compared with the unsubstituted compounds when tested against *S. mansoni* in mice.

activity at 50 mg./kg. There is once more similar activity at 200 mg./kg. to that of the unsubstituted compounds at 25 mg./kg. (Fig. 3). The secondary amines of this series (Table I*E*) do not show the marked superiority over the primary amines observed in the corresponding methyl substituted series. The tertiary amines (Table I*F*) resemble the primary amines with only the compounds $n=$ 7 to 9 showing marked activity at maximum dosage, and, like the primary amines, differ from the corresponding methyl series in that activity is only apparent at greater chain-length.

Other Substituents.—Primary amines, with various other substituents, were also tested (Table I*G*). In the (I; $R^2=Cl$, $R^3=H$) series, none of the compounds $n=3$ to 10 shows activity except $n=8$, which is slightly active. For (I; $R^2=$ H, $R^3=Cl$) the only compound tested, $n=6$, is inactive, and for (I; $R^2=NH_2$, $R^3=H$) the compounds $n=4$ to 7 are all inactive.

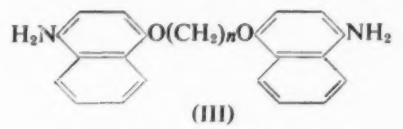
There is considerable evidence that an early step in the metabolism of aromatic amines is the substitution of a hydroxyl group into the para-position to the amino-group, or if this position is blocked, as in our compounds, substitution into the ortho-position. Consequently, compounds of the series (I; $R^2=H$, $R^3=OH$) might represent metabolites of the symmetrical diaminodiphenoxyalkanes, and it was therefore of interest to examine this type. The two primary amines, $n=6$ and 7, are inactive at 200 mg./kg. orally, and also at 50 mg./kg. by parenteral routes; the isomeric compounds (I; $R^2=OH$, $R^3=H$) are also inactive for $n=5$ and 7. These results suggested that the normal metabolic processes might lead to inactive compounds, and that blocking of the free ortho-positions might prevent this dystherapeutic effect and have a favourable influence on activity, but all the compounds ($n=3$ to 8) of the general formula (II)



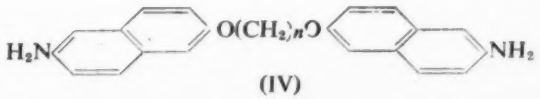
(II)

that were tested are devoid of activity at 200 mg./kg.

The naphthalene compounds (III; $n=5$, 7) and



(III)

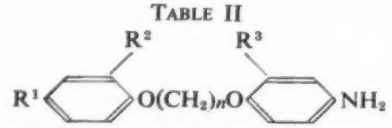


(IV)

(IV ; $n=2$ to 4), of which the former may be regarded as substituted diaminodiphenoxyalkanes, are all inactive at 200 mg./kg.

Table II gives details of the effect of substituting a methyl group into one ring only of (I). For a chain-length of four carbon atoms and with *p*-amino-groups in both rings there is a marked fall in activity as each ring is substituted in turn with a methyl group (384C50, 496C55, 117C53). For a chain-length of seven carbon atoms introduction of a methyl group into one ring reduces activity, but the compound with methyl groups in both

TABLE II



Compound Number	*n*	R^1	R^2	R^3	Unit Dose (mg./kg.)	Worms Killed %
325C54	4	NO_2	H	H	200	100
					50	76
495C55	4	NO_2	H	CH_3	200	100
					50	98
384C50	4	NH_2	H	H	200	99
					50	66
					25	17
496C55	4	NH_2	H	CH_3	200	100
					50	8
117C53	4	NH_2	CH_3	CH_3	200	79
					50	1
254C53	7	NO_2	H	H	200	97
					50	21
48C54	7	NO_2	H	CH_3	200	100
					50	0
153C51	7	NH_2	H	H	50	100
					25	96
					15	42
120C54	7	NH_2	H	CH_3	200	100
					50	29
107C53	7	NH_2	CH_3	CH_3	50	Toxic

rings is highly toxic (153C51, 120C54, 107C53). With a methyl group in one ring and a nitro-group in place of the amino-group in the other the results are anomalous, for whereas the 7-chain compounds have the expected order of activity (254C53> 48C54), with the 4-chain the methyl compound (495C55) is more active than the unsubstituted compound (325C54), or indeed than the unsubstituted diamine (384C50).

Discussion

The striking dystherapeutic effect of further substitution of the aromatic rings of the diaminodiphenoxyalkanes is apparent, particularly when the substituent is *ortho* to the amino-group. This class shows complete loss of activity for all but two examples (186C53 and 187C53) (Table I*A*), which show slight activity at maximum dosage, and this suggests that some essential form of combination between the amino-group and substrate is prevented or hindered by the adjacent substituent and, in view of the varied polar nature of the groups studied, this effect may well be steric. Very great loss of activity also occurs when the substituent is in the ortho-position to the ether linkage, and although now the loss is not usually complete it is tempting to ascribe a similar cause also to this dystherapeutic effect. The implication is that at some stage cleavage of the drug occurs with liberation of a phenolic group, which in turn combines with the substrate, such combination being hindered by ortho-substituents. The parallelism in activity observed between the more active of the substituted and the corresponding unsubstituted compounds (Figs. 1–3), and the usually additive effect of substitution of each ring in turn (Table II) would not be incompatible with such a notion, but no evidence has yet accrued that any possible cleavage products of the active compounds have schistosomicidal activity.

Summary

1. Substitution of CH_3, OCH_3, OH, Cl, or NH_2 into the aromatic rings of the symmetrical diaminodiphenoxyalkanes invariably results in a great reduction in activity against *Schistosoma mansoni*, loss of activity usually being complete when a substituent ortho to the amino-group is present in both rings.

2. The most active of the compounds in which both rings are substituted are the primary amines with methoxyl ortho to the ether linkage, and secondary amines with methyl similarly placed, but even these are only as active at 200 mg./kg. as are the unsubstituted compounds at 25 mg./kg.

3. When one ring only is substituted with methyl the activity is intermediate between that of the unsubstituted and the disubstituted compounds, except for a nitro-amine substituted with methyl in the basic ring, which has high activity.

We are indebted to our colleagues Drs. Caldwell, Gorvin, and Raison for some of the compounds referred to, and for valuable technical assistance to Mr. R. Nightingale with the chemical work and to Mrs. Rosemary Richards with the biological work.

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A MODIFICATION OF RECEPTOR THEORY

BY

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It is usually supposed that a drug combines with some part of a tissue as a necessary step in producing its action on that tissue. This view was expressed by Langley in 1878 while discussing experiments with pilocarpine and atropine. He later referred to "receptive substance" (Langley, 1905) and subsequently the term "receptor" has been widely used to express the same concept. Clark (1937a) attempted to put the concept on a quantitative basis by applying the adsorption isotherms of Langmuir which were derived from the application of the mass laws to the adsorption of gases on metal surfaces. The derivation of the equations as they apply to drugs in solution, and receptors or active patches in tissue, may be put as follows: Drug molecules combine with receptors at a rate which is proportional to the concentration of molecules in solution and to the number of free receptors. The resulting complex breaks down at a rate proportional only to the number of complexes. Thus the rate of combination is $k_1A(1-y)$ and the rate of dissociation is k_2y, where k_1 and k_2 are constants, A the concentration of drug, and y the proportion of receptors occupied by the drug. When the two rates are equal we have, putting K for $\frac{k_1}{k_2}$,

$$KA = \frac{y}{1-y} \quad \ldots\ldots\ldots\ldots\ldots\ldots 1$$

When $y=0.5$, then

$$A = \frac{1}{K} \quad \ldots\ldots\ldots\ldots\ldots\ldots 1a$$

Equation 1 therefore relates the concentration of drug and the proportion of receptors which are occupied by drug molecules at equilibrium. Clark, however, went a step further than this and, by taking y as the response, used equation 1 to relate the response of the tissue to the concentration of drug. Thus there was in Clark's treatment the implicit assumption that the percentage of receptors occupied is equal to the percentage response of the tissue. When 50% of the receptors are occupied there is 50% of the possible response; when the response is maximal all the receptors are occupied.

Clark sought to show that the relation between concentration of drug and the effect produced did in fact agree with equation 1, and his apparent success was some justification for the assumption. The two situations he investigated were (a) the contraction of the isolated frog rectus abdominis produced by acetylcholine, and (b) the inhibition produced by acetylcholine of the contraction of strips of electrically stimulated frog ventricle. These tests have a common disadvantage in that both tissues contain enzymes which destroy acetylcholine, so that the concentration of drug in equilibrium with the receptors is less than that applied externally, and, though the applied concentration is known, that in the tissue is not. The receptors, indeed, may not all be in equilibrium with the same concentration of acetylcholine; a gradient is presumably established with a high concentration near the surface and a lower concentration at the centre of the tissue—because the further the acetylcholine penetrates the greater is its chance of being destroyed. Such a gradient would produce a log-concentration-effect curve flatter than the true curve. Furthermore, it cannot be assumed that the ratio of the applied concentration to the effective concentration (or concentrations) is constant for different applied concentrations. A block of cholinesterase activity would therefore be expected to alter the slope of log-concentration-effect curves; Webb (1950) has shown that this is so, and that the change of slope is considerable. Calculation from his graphs shows that, when the cholinesterase of isolated rabbit auricles is blocked with physostigmine, the slope of the acetylcholine log-concentration-effect curve is about 10 times steeper than on normal auricles (see Table I). An attempt has been made to determine the relation between log concentration and effect for acetylcholine and the frog rectus after blocking the cholinesterase with TEPP so that the size of the effect could be assessed in one of the

systems used by Clark. This proved to be difficult, since the response of the rectus, always slow, became very much slower after TEPP, and the contractions continued to increase for an hour after acetylcholine was applied. The action of carbachol on normal recti was similarly slow. It was therefore impossible to get an accurate estimate of slope, but it was evident that the increase of effect with increased concentration was greater than required by equation 1. In any case it was clear that reliable evidence in support of any particular equation would be difficult to obtain from this tissue.

Clark discussed several other drug-tissue systems, but he was mainly concerned to show that a graded response was produced over a wide range of concentration and, in most of the examples he quoted, the slopes of the log concentration curves are not in accord with equation 1. A simple way of reducing data about concentration-effect curves to a single figure which is independent of any theory of drug action, and which can therefore be used to test any particular theory, is to use, as Clark did, the ratio of concentrations causing specified effects. Clark used the ratio for 84% and 16% of the maximum contraction; but a narrower range is easier to cover, and there is no need to restrict the ratio measured to any particular pair of responses. Evidence from which such ratios can be calculated is rare, because the maximum response is seldom recorded, but a few examples are shown in Table I. Some of these ratios represent new data for acetylcholine and histamine on guinea-pig ileum; details are given in the section on results. Furchgott and Bhadrakom commented on the theoretical implication of the slope, and considered that their results agreed with equation 1; the ratio given in Table I expresses this agreement. The other ratios in Table I have been calculated from published graphs: they are not in agreement with equation 1.

A variety of slopes can be accounted for by modifications of equation 1 obtained by postulating that a molecule of drug combines with either less or more than one receptor. The latter possibility may be true in some instances, but the postulate is unlikely to be generally correct—particularly where fractions are involved. Equation 1 appears to be the most likely relation between concentration of drug and the percentage of receptors occupied, but Table I shows that there is no experimental justification for supposing that it represents a general relation between concentrations of drug and the response of the tissue. It therefore becomes necessary to consider possibilities other than the one assumed by Clark for the relation between the proportion of receptors occupied and the response, and we can broaden the application of the receptor theory by the following hypotheses:

(1) A maximum effect can be produced by an agonist when occupying only a small proportion of the receptors.

(2) The response is not linearly proportional to the number of receptors occupied.

(3) Different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses. This property will be referred to as the efficacy of the drug.

The concept of efficacy, which may vary from zero to a large positive value, is at variance with Clark's (1937c) view of the action of a drug at a receptor as all or none; an agonist (Reuse, 1948) causes an effect and an antagonist causes no effect so that the activity of a drug of either kind is simply a measure of its affinity for the receptors. According to hypothesis 3, however, this remains true only for antagonists (which have zero efficacy): the activity of agonists is the product of their affinity and their efficacy. A drug with very low efficacy may produce a response which is less than the maximum, even when it is occupying nearly all the receptors—and because it is occupying the receptors, it diminishes the action of a drug with high efficacy added simultaneously. Compounds with such a low efficacy that they possess properties intermediate between agonists and antagonists will be called " partial agonists." (See p. 384.)

Ariëns and Groot (1954) have also observed substances with these intermediate properties, and they ascribe this to a low " intrinsic activity." Ariëns and Groot, however, continue to assume that all normal agonists have the same " intrinsic activity " and their concept is more limited in application than hypothesis 3.

The above hypotheses account for the progressive variation in properties in a series of alkyltrimethylammonium salts.

The lower homologues have acetylcholine-like activity whereas the higher members have an atropine-like blocking action (Raventós, 1937a and b; Clark and Raventós, 1937). It was expected that the members of such a simple homologous series would display some progressive change in their affinity for the receptors, and consequently, if Clark was correct, the reciprocals of the concentrations of the agonists causing a 50% response (see equation 1a) would form part of the same progression as the affinity constants of the antagonists. The demonstration of a sharp discontinuity be-

tween the two sets of constants, such that the agonists are effective in lower concentration than the antagonists, would, however, constitute good evidence for hypothesis 1. Raventós showed that the acetylcholine-like activity of the lower homologues varied rather irregularly, but calculation from his data suggested that the expected discontinuity between agonist and antagonist activity did occur. The nature of the evidence did not allow a firm conclusion. It was therefore decided to re-examine the compounds, with the intention of obtaining accurate values for the affinity constants of the first 4 or 5 antagonists, and to compare these with the reciprocals of the concentrations of the agonists which cause a 50% contraction.

METHODS

A guinea-pig (usually weighing 150–200 g.) was killed by a blow on the head and bled. The terminal 3–4 cm. (avoiding the patch of lymph tissue) of the ileum was suspended in Tyrode solution maintained at $37.0 \pm 0.1^\circ$ C. It was attached to an isotonic frontal writing lever giving a magnification of 4 and a load of 0.5 g. The bath was connected to coils of glass tubing so that the fluid in the bath could be changed by upward displacement and overflow, either by Tyrode alone or by Tyrode containing drugs at predetermined concentrations. The volume of the bath was about 2.5 ml. and that of the coils 10 or 20 ml., so that sufficient solution could be run through the organ bath to effect a complete exchange (verified by using strongly coloured solution) without exposing the muscle to air and without cooling (less than 0.1° C.). Events in the bath were controlled by an automatic apparatus similar to that described by Schild (1947). Contractions were produced either by agonist solutions replacing the Tyrode or by additions from a tuberculin syringe at a signal from the apparatus. In addition to the uniselector controlling the cycle of operations a second uniselector could be used to perform an assay ; 4 different agonist solutions being used in varying order to produce the contractions: up to 48 contractions could be used in the assay (12 groups of 4 arranged in 3 Latin squares). A similar arrangement has been used by Boura, Mongar, and Schild (1954).

RESULTS

Slopes of Acetylcholine and Histamine Concentration-Effect Curves.

These experiments were performed primarily to investigate the action of antagonists, but concentration-effect curves were obtained before the antagonist was applied and these are relevant here. As soon as the ileum was put in the bath a small concentration of agonist (usually 2.5 or 5.0 ng./ml. acetylcholine or 5 or 10 ng./ml. histamine) was applied to the muscle for 10 or 15 sec. and then washed away. This was repeated at intervals of 1½ min. until the responses became steady: this usually took about an hour. About 4 concentrations of agonist differing by a factor of 2 were then tested: each concentration was used for 3 or 4 successive contractions before being replaced by another. At least two such groups of contractions were obtained for each concentration and the order in which the concentrations were tested was varied. The maximum contraction was obtained at the end of the experiment, after the antagonist being tested had been washed from the bath but sometimes at a time when the muscle was still to some extent under the influence of the antagonist. A large dose of agonist (about 100 times that giving a 50% response) was, however, used to obtain the maximum contraction, and there appeared no reason to suppose that the response was different from that which would have been produced before the antagonist was tested. The contractions were measured to the nearest 0.5 mm., plotted against log concentration, and a curve fitted by eye. The concentrations producing 20, 50 and 80% contractions were read from this curve. The results from 13 experiments with acetylcholine have been analysed. In only 8 of these were responses less than 20% of the maximum obtained, so that in five experiments the dose ratio could only be calculated for the 80%/50% response ratio. All the results have been included in Table I, since a biased sample could result if only the experiments giving a wide range of responses were presented. (Only four concentrations, covering a 16-fold range, were usually tested so that 20% and 80% responses could not both be obtained in a preparation in which the dose ratio for these responses was more than 16 ; and would often not be obtained if the ratio was only slightly less than 16.) The experiments with histamine were done on ileum suspended in Locke solution containing 10^{-7} atropine. The results of 38 experiments have been examined ; only 13 included both 20% and 80% responses, 10 did not include the 80% and 15 did not include the 20% response. The slopes varied considerably, particularly those for histamine. The geometric means and the standard deviations were calculated from the logarithms of the ratios. These means and the values of the ratios corresponding to one standard deviation above and below the mean (being the values between which two-thirds of the observations would be expected to lie) are shown in Table I. None of the acetylcholine slopes and only a small proportion of those for histamine were as shallow as required by equation 1. The variations in slope are probably genuine and not due to error, and this in itself would show that

TABLE I

SLOPES OF CONCENTRATION-EFFECT CURVES

The numerals in column A give values of the ratio—

$$\frac{\text{concentration of drug producing 80\% response}}{\text{concentration of drug producing 20\% response}};$$

and cols. B and C the ratios for the 80%/50%, and the 50%/20% responses, respectively. The values expected from equation 1 are 16, 4 and 4, in cols. A, B, and C respectively

Authors	Slopes A	Slopes B	Slopes C	Drugs, and System on which Tested
Gaddum, 1926	1·8			Adrenaline: contraction of rabbit uterus
Raventós, 1937	2			Acetylcholine: inhibition of amplitude of beat of electrically stimulated frog auricle
Schild, 1947		2·5		Histamine: contraction of guinea-pig ileum
Webb, 1950	56			Acetylcholine: reduction in amplitude of spontaneously beating rabbit auricles
	5·4			Carbachol: ditto
	5·3			Acetylcholine: ditto with physostigmine 10^{-6}
			12·9	Acetylcholine: reduction of rate of beating of isolated rabbit auricles
	3·7			Acetylcholine: ditto after treatment with physostigmine 10^{-6}
Van Maanen, 1950	30			Acetylcholine: contraction of frog rectus
Stephenson (see text)	~4			Ditto after TEPP
Furchgott and Bhadrakom, 1953	16			Adrenaline: contraction of rabbit aorta strips
Stephenson (see text)	7·7 (8)	2·7 (15)	3·0 (8)	Acetylcholine: contraction of guinea-pig ileum. Mean and (no. of observations)
	6·4, 9·2	2·2, 3·4	2·6, 3·4	Range ($P=0·67$)
	11·1 (13)	3·2 (23)	3·4 (28)	Histamine: contractions of guinea-pig ileum. Mean and (no. of observations)
	6·3, 19·5	2·5, 4·0	2·5, 5·3	Range ($P=0·67$)
(see Fig. 1)	10			Butyl trimethylammonium: contraction of guinea-pig ileum

equation 1 does not represent the dose-effect curve. If, however, the variation is interpreted as random error, five of the six mean estimates of slope are significantly different from the values expected from equation 1. For the 50%/20% ratio for histamine P lies between 0.2 and 0.1: for the 80%/50% ratio for histamine $P<0.05$, and for the other ratios $P<0.001$.

Alkyltrimethylammonium Salts (Alkyl-TMA)

The action of the first six members of the series, tetramethylammonium (methyl-TMA), ethyl-TMA, propyl-TMA, butyl-TMA, amyl-TMA and hexyl-TMA, appeared to be fairly straightforward, and a series of (2 and 2) dose assays were made to compare their potencies. Ileum was set up as already described and assays started when the responses became regular. To facilitate detection of any difference of slope a dose ratio of 4 was at first used. However, the contractions produced by the high concentrations were observed to depress the subsequent response to a small concentration, thereby increasing both the variation and the apparent slope. Deviation from parallelism was not seen except with propyl-TMA, which sometimes had a steeper slope than amyl-TMA, and most of the assays were done with a dose ratio of 2. The effect of the first 3 members was reduced by the addition of hexamethonium to the Tyrode; 50 mg./l. and 100 mg./l. were tested and the latter concentration adopted as a routine. This concentration of hexamethonium produced a small continuous activity which was prevented by mepyramine and was presumably due to histamine liberation. Mepyramine 10 μg./l. was therefore also added to the Tyrode in all subsequent experiments. Amyl-TMA, the most active of the series, was used as the standard in each assay. After preliminary tests, 33 assays on 12 pieces of ileum (each from a different guinea-pig) were performed. Each assay consisted of 10 or 12 groups of 4 contractions. The results are summarized in Table II. The assays performed in the presence of 100 mg./l. hexamethonium gave very uniform results except for those of propyl-TMA, where the standard deviation of the population of assays ($n=6$) was about 15% of the mean. With butyl-TMA the standard deviation was only about 1.7% of the mean. An experimental error of at least this size is to be expected so that there can be little if any variation due to differences between guinea-pigs.

The change in relative activity caused by hexamethonium indicates that a nicotine type of action contributes to the response obtained with methyl-, ethyl-, and propyl-TMA. The fourfold change in activity with propyl-TMA suggests that in the absence of hexamethonium the contraction of the ileum is largely due to nicotinic rather than muscarinic activity.

The concentration of agonist required to produce a 50% response varies both from one piece of ileum to another and with one piece at different times. If the ileum is handled with great care during preparation, is not picked up with forceps,

not subjected to a pressure of more than 1 or 2 cm. of water when the lumen is washed out, and not stimulated with high concentrations of agonist in the early insensitive period, the variation is not wide. Even with great care there remains a residual variation, and values for concentrations producing 50% effect can only be statistical means. The responses to amyl-TMA for that assay during which each ileum showed its greatest sensitivity were calculated as a percentage of its maximum; the concentration for 50% effect was obtained by interpolation or a short extrapolation. The maximum contraction was only obtained for 7 of the 9 pieces of ileum on which assays were done in the presence of 100 mg./l. hexamethonium. For these 7 pieces of ileum the concentration of amyl-TMA required for a 50% contraction was $5.8 \pm 0.9 \times 10^{-7}$M (mean $\pm$SE, $n=7$). The corresponding concentration for each of the other compounds was calculated from the ratios in column 3 of Table II and the results are shown in column 4 of that table.

The transition from agonist to antagonist activity in the higher members of the series was not as simple as expected; some compounds showed intermediate effects. This should perhaps have been foreseen, since Raventós (1937) made passing reference to hexyl-TMA and octyl-TMA producing effects, on the frog heart and rat ileum respectively, which could not be increased by increasing the concentration. In the present experiments on guinea-pig ileum, heptyl-, octyl-, nonyl-, and decyl-TMA all produced contractions, but increasing the concentration failed to produce the maximum contraction. The largest contraction which could be obtained decreased as the length of the carbon chain increased, and decyl-TMA was only able to produce a contraction about 15% of the maximum produced by acetylcholine and by the alkyl TMA ions up to hexyl-TMA. The size of the largest contraction produced by any of these four partially active compounds varied from preparation to preparation and from time to time. This variation appeared to parallel the variation in sensitivity to normal agonists, the difference being, of course, that lowered sensitivity to a normally active compound did not reduce the size of the maximum contraction but only required an increased concentration to produce it.

In Fig. 1 the contractions have been expressed as a percentage of the maximum obtainable with a true agonist. All the observations were made on the same piece of ileum over a period of about 5 hours. During this time there were small changes in sensitivity, but the relative positions of the curves give a good idea of the differences between

TABLE II

COMPARISON OF ACTIVITY OF ALKYL TRIMETHYL-AMMONIUM IONS ON GUINEA-PIG ILEUM

Assays of five compounds against amyl-TMA. Col. A gives results obtained in the absence of hexamethonium; cols. B and C those obtained in the presence of 50 mg./l. and 100 mg./l. hexamethonium respectively, together with mepyramine maleate 10 μg./l., except in the one assay marked † in col. C. The numerals in cols. A, B, and C are for individual assays except in two cases, where the mean and standard deviation, based on the number of assays shown in parentheses, are given. The numerals in the last column are calculated from the means of the results in the preceding column and the mean of the observed values for amyl-TMA. In all assays, except where otherwise indicated, the ratio of high doses to low doses was 2

Drug	Activity as Percentage of Amyl-TMA			Concn. Producing 50% of Maximum Contraction ($M \times 10^6$)
	A	B	C	
Methyl-TMA	*2·08	1·56, 1·54, 1·38	1·48, 1·30, 1·25	43·5
Ethyl-TMA	*1·50	1·41, 1·25	1·17, 1·13, 1·07	52·0
Propyl-TMA	*1·15, *1·12		(6) 0·299 ± 0·08	195
Butyl-TMA	*49·0, *44·9, *42·0	*45·0, 44·5	(5) †43·8 ± 0·34	1·33
Amyl-TMA	100	100	100	0·582 ± 0·092
Hexyl-TMA	—	—	22·7, 24·3	2·48

* Assays done with dose ratio of 4.
† Includes one assay done with dose ratio of 4.

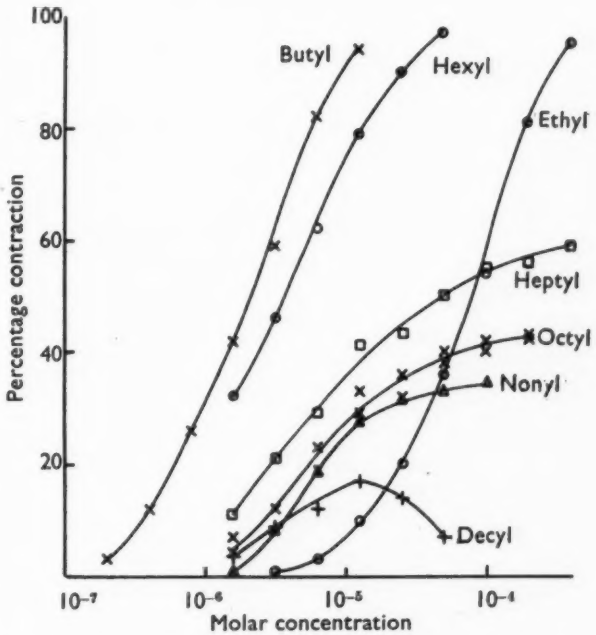

FIG. 1.—Concentration-effect curves for some alkyl-TMA ions on guinea-pig ileum. The contractions were produced by additions to the bath to give the final concentrations indicated; each concentration was left in contact with the ileum for about 15 sec. and then washed away. All the points for a given substance were obtained in consecutive contractions, starting with a low dose which was progressively doubled until the response range was covered. This was done twice with octyl-TMA and both sets of points are plotted.

the substances. The highest two doses of decyl-TMA were sufficient to reduce the surface tension of water so that there was some frothing ; this may be associated with the fact that these doses produced less effect than the smaller dose.

The fact that these four compounds were neither true agonists nor true antagonists made impossible the comparison of potency of very closely related agonists and antagonists which had been envisaged. It was evident, however, that the actions of these compounds were consistent with, and therefore supported, the hypotheses under consideration. If different agonists have different efficacies then compounds with very small efficacies may be envisaged, and it is suggested that the observed actions of heptyl-, octyl-, nonyl-, and decyl-TMA are due to their having efficacies which are so low that, unlike the more familiar agonists, they must occupy a large proportion of the receptors in order to produce a contraction. Evidence in support of this explanation was obtained when one of the active members of the series was tested simultaneously with one of the partially active substances. Fig. 2 shows the effect of adding a dose of butyl-TMA which alone produced a near maximum contraction with a dose of octyl-TMA which alone produced a much smaller contraction. The two together produced very little more effect than octyl-TMA alone, indicating that octyl-TMA was occupying the receptors to the exclusion of butyl-TMA. Even four times the concentration of butyl-TMA added with the octyl-TMA did not produce an effect as large as the single dose of butyl-TMA acting alone. The four substances heptyl- to decyl-TMA thus seem to possess a type of action which is a mixture of agonist and antagonist effects, and they will—as pointed out on p. 380—be referred to as partial agonists.

FIG. 2.—Guinea-pig ileum. Tyrode with hexamethonium iodide 100 mg./l. and mepyramine maleate 10 μg./l., at 37° C. Contractions produced by adding octyl-TMA and butyl-TMA to give the following concentrations in the bath. At O, 4×10^{-4}M-octyl-TMA; at B, 1.6×10^{-5}M-butyl-TMA; at O+B the same concentrations acting together; at O+2B and O+4B the same concentration of octyl-TMA together with 3.2×10^{-5}M- and 6.4×10^{-5}M-butyl-TMA respectively. Recording drum stopped simultaneously with wash-out.

More elaborate experiments of the type shown in Fig. 2 made it possible to estimate the proportion of the receptors which were occupied by given concentrations of partial agonists and hence to calculate their affinity constant.

To interpret these experiments the following theoretical relations of the response, R, the efficacy, e, and the proportion of receptors occupied, y, are advanced. Since R is not proportioned to y (hypothesis 2) it is useful to introduce the quantity S, which may be regarded as the stimulus given to the tissue, and which is defined as the product of e and y. We can write $R=f(S)$ signifying that for any value of S the value of R is determined, but that we do not know how the two are related. We have by definition $S=ey$, therefore from the mass law, equation 1, we have

$$S=e\frac{KA}{1+KA} \quad \ldots\ldots\ldots\ldots\ldots 2$$

For an active agonist, where e is high and only a small proportion of the receptors are occupied, KA is small compared with 1 and equation 2 reduces to

$$S=eKA \quad \ldots\ldots\ldots\ldots\ldots 2a$$

The efficacy e can have any positive value ; there will presumably be some practical limit, but no theoretical limit can be imposed. It is convenient to adopt the convention that $S=1$ for a 50% response. When this is done a drug which occupies all the receptors to produce an effect which is only 50% of the true maximum has an efficacy $e=1$, since $S=ey$.

Let A_1, A_2, and A_3 be concentrations of an active agonist (with efficacy e_a) and let P be the concentration of a partial agonist (efficacy e_p) such that A_1 produces the same contraction as P and that A_2 produces the same contraction as P and A_3 acting simultaneously. We have, for the two sizes of contraction, $R_1=f(S_1)$ and $R=f(S_2)$. If x is the proportion of receptors occupied by the partial agonist (which will be the same whether A_3 is present or not since, according to hypothesis 1, an active agonist occupies a negligible proportion of the receptors to produce an effect) we have $S_1=e_a x$ and also, using equation 2a, $S_1=e_a K_a A_1$ so that

$$e_p x=e_a K_a A_1 \quad \ldots\ldots\ldots\ldots\ldots 3$$

again $S_2=e_a K_a A_2$ and, if values of S are assumed additive, $S_2=e_p x+e_a K_a A_3(1-x)$ since the partial agonist occupies a significant proportion of the receptors. We therefore have

$$e_a K_a A_2=e_p x + e_a K_a A_3(1-x) \quad \ldots\ldots\ldots 4$$

Combining equations 3 and 4

$$e_a K_a A_2=e_a K_a A_1 + e_a K_a A_1(1-x)$$

$$1-x=\frac{A_2-A_1}{A_3} \quad \ldots\ldots\ldots\ldots\ldots 5$$

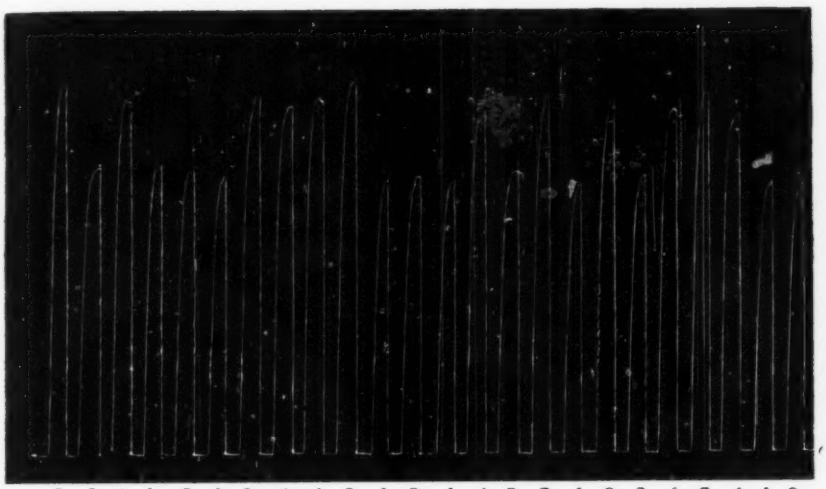

FIG. 3.—Guinea-pig ileum, conditions as in Fig. 2. Contractions produced by filling the bath for 15 sec. with solution containing: 1, 1.4×10^{-6}M-butyl-TMA; 2, 2.8×10^{-6}M-butyl-TMA; 3, 6.4×10^{-5}M-octyl-TMA; 4, 6.4×10^{-5}M-octyl-TMA + 5×10^{-6}M-butyl-TMA. The complete assay consisted of 48 contractions; the first 24 are shown.

Fig. 3 shows the first half of an experiment arranged to find values to insert in equation 5. Contractions of guinea-pig ileum were produced by four different solutions; 1, which contained 1.4×10^{-6}M-butyl-TMA; 2, which contained 2.8×10^{-6}M-butyl-TMA; 3, which contained 6.4×10^{-5} M-octyl-TMA; and 4, which contained both 6.4×10^{-5}M-octyl-TMA and 5.0×10^{-6}M-butyl-TMA. The concentrations of butyl-TMA were chosen so that the contractions produced by solution 1 were similar to those produced by 3, and those produced by 4 were similar to those produced by 2. Over small ranges the response to butyl-TMA (as with other agonists) may be regarded as linearly related to the logarithm of the concentration, and this relation was used to calculate from the measured responses that concentrations of 1.482×10^{-6}M- and 2.462×10^{-6}M-butyl-TMA would have produced contractions equal in height to those produced by solutions 3 and 4 respectively. Inserting these values in equation 5 indicates that for 6.4×10^{-5}M-octyl-TMA the proportion of receptors occupied is 0.804 and equation 1 indicates that 1.56×10^{-5}M would occupy 50% of the receptors or $K_{Oct}=6.4\times10^{4}$.

Experiments similar to that described above were done with different concentrations of heptyl-, octyl-, and nonyl-TMA. The range over which the concentration could reasonably be varied was not very wide. If the partial agonist occupied less than 20% of the receptors the experimental error became disproportionately great, while if much more than 80% were occupied the concentration A_3 of butyl-TMA had to be large and the contractions persisted after washing and upset the assay. Within these limits the calculated values of x agreed well with the mass law relation (equation 1). This was clearest with octyl-TMA and nonyl-TMA, and the results of *all* the tests made with these substances are shown in Table III. These results suggest that equation 5 is valid.

Most of the tests with heptyl-TMA were done while the procedure was being established and the results were more variable. Tests with low concentrations of heptyl-TMA gave slightly higher values for K_{Hep} than did tests with higher concentrations. Most of the tests were done with intermediate concentrations so that the total variation was not large. With most pieces of ileum the spontaneous activity became quite marked when only small contractions were induced, and only twice did it seem feasible to do assays of this type with decyl-TMA. These two experiments, however, gave the reasonably consistent values of 1.74 and 2.02×10^{-5} for K_{Dec}. The values for K for the four partial agonists (which are listed in Table V) increase steadily with the increase in chain

TABLE III
RESULTS OF ADDITION EXPERIMENTS

Octyl-TMA			Nonyl-TMA		
Concn. M × 10^5	% of Receptors Apparently Occupied	Concn. to Occupy 50% Receptors $= \frac{1}{K_{Oct}}$ M × 10^5	Concn. M × 10^5	% of Receptors Apparently Occupied	Concn. to Occupy 50% Receptors $= \frac{1}{K_{Non}}$ M × 10^5
0·4	22·1	1·41	0·4	30·15	0·93
			0·4	39·2	0·62
1·6	46·8	1·81			
1·6	56·2	1·25	1·6	63·1	0·94
1·6	53·0	1·42	1·6	63·2	0·93
1·6	47·7	1·75	1·6	63·4	0·92
			1·6	67·8	0·76
6·4	78·6	1·74	1·6	62·7	0·95
6·4	77·6	1·84	1·6	63·8	0·96
6·4	80·4	1·56	1·6	70·6	0·67
6·4	79·9	1·6			
			6·4	86·4	1·01
				85·6	1·08
Means ± S. E.		1·60 ± 0·068 × 10^{-5}M			0·89 ± 0·043 × 10^{-5}M
Corresp. values of K		0·625 × 10^5			1·125 × 10^5

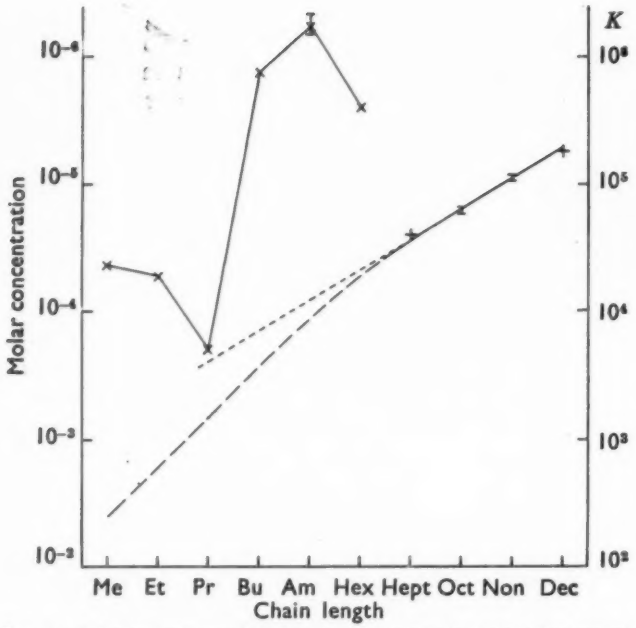

FIG. 4.—Relation between chain length and activity in the alkyl trimethylammonium series. The points plotted X–X for the first 6 members of the series are the concentrations which produce 50% of the maximum contraction (data from Table II). The points for the next 4 members are the estimates of the concentrations which occupy 50% of the receptors. Standard errors are shown in two instances. The ordinate on the left is concentration (log scale) and that on the right is the reciprocal concentration. On the right the points plotted for the partial agonists become affinity constants. If Clark's assumption is correct the points plotted for the agonists also represent affinity constants. The extrapolations are discussed in the text.

length of the molecules. In Fig. 4 the mean values for K are plotted on a log scale together with the figures from Table II of the concentrations of the agonists which produce 50% of the maximum contraction. The straight line drawn through the affinity constants passes, when extrapolated, well below the points for 50% response for the agonists other than propyl-TMA. It would seem likely that the affinity constants for the true agonists also lie on or near this line. It is evident, however, that a straight line does not give an accurate estimate of the affinity constant of propyl-TMA, since the value indicated is little less than the reciprocal of the concentration which produced a 50% response; if the concentration at which 50% of the receptors are occupied is, in fact, only slightly greater than the concentration which produces 50% response, then the occupation of all the receptors would not produce a maximum response (see discussion). In fact this substance did produce the maximum contraction. The position is complicated by the possibility that part of the response of the ileum to propyl-TMA was due to a nicotine effect despite the presence of 100 mg./l. hexamethonium. With 500 mg./l. hexamethonium propyl-TMA still produced maximum contractions, but the slope of the log-concentration-effect curve was reduced: these were not good experiments, since the ileum did not behave well with this high concentration of hexamethonium. With the usual 100 mg./l. hexamethonium the nature of the contraction due to propyl-TMA was different from that of the other members of the series (and ACh); it reached its peak in a few seconds and then declined quite quickly—perhaps halfway to the base line in 15 sec. It was probably for this reason that propyl-TMA did not give additive effects with butyl-TMA. This lack of addition was at first misinterpreted as showing that quite small concentrations of propyl-TMA occupied an appreciable proportion of the receptors. With higher concentrations of propyl-TMA, however, the percentage of receptors apparently occupied did not increase in accordance with equation 1, and it became clear that the value of K for propyl-TMA was certainly less than that indicated by the straight line of Fig. 4.

If the affinity constants of the short chain compounds increase in the regular way shown by the partial agonists, the surprisingly low muscarinic activity of propyl-TMA (Table II) must be due to a relatively low efficacy. This was confirmed in pieces of ileum in which the sensitivity became low in experiments prolonged for 2 or 3 days. It was observed that propyl-TMA lost its ability to produce the normal maximum contraction though this was still readily obtained with both ethyl- and butyl-TMA. Hexyl-TMA was also unable to produce maximum contractions in these tests. It is possible to estimate relative efficacies from the height of maximal contractions where these are less than the true maximum (see discussion), and it appeared that the efficacy of hexyl-TMA was 4–5 times that of propyl-TMA. The curved line in Fig. 4 extending from the estimated affinity constants of the partial agonist has been drawn so that the affinity constant indicated for propyl-TMA corresponds to an appropriate value for efficacy. It will be assumed that the affinity constants of all the compounds lie on this line.

The gap between this line and the points for 50% response supports the hypothesis that active compounds of this series, and by inference other similar substances, including acetylcholine, produce their effects when only a small fraction of the receptors are occupied.

An interesting effect was observed when the partial agonists acted on the ileum in the presence

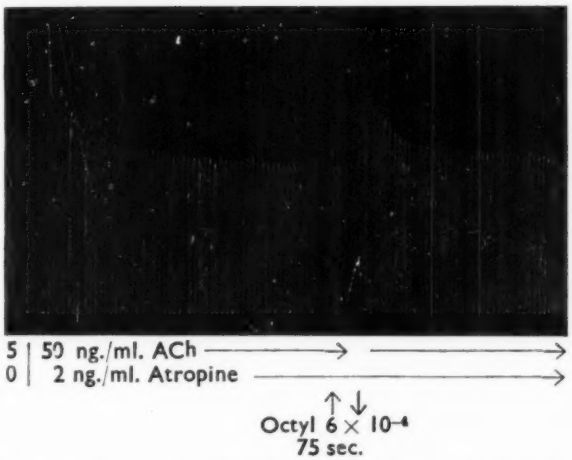

FIG. 5.—Guinea-pig ileum, conditions as in Fig. 2. Regular contractions produced by acetylcholine acting for 15 sec. at 1 min. intervals. The first 6 contractions were produced by ACh 5 ng./ml. After the 6th contraction the ACh was washed from the bath with Tyrode containing atropine 2 ng./ml. (in addition to hexamethonium and mepyramine) and this concentration was then maintained continuously: subsequent contractions were produced by ACh 50 ng./ml. At the vertical arrow octyl-TMA was added to give a concentration of 6×10^{-4}M and left in the bath for 75 sec.: ACh was then applied 45 sec. later and at 1 min. intervals.

of atropine. The dose had, of course, to be increased to produce a contraction, but when sufficient was applied the contraction was much slower than usual and the partial agonist had to be left in the bath for about 100 sec. to allow the contraction to develop fully. This compares with the 5 or 10 sec. which is usual for the partial agonists in the absence of atropine and for normal agonists whether atropine is present or not. When such a slowly developing contraction was elicited during an interruption in a series of acetylcholine contractions, a sensitization of the ileum was observed when the acetylcholine contractions were resumed. The size of the contractions then declined again at a rate very similar to that of the decline when atropine was first applied. This similarity is evident in the experiment shown in Fig. 5, where the atropine produced a 10-fold reduction in sensitivity to acetylcholine. These effects were only seen with the partial agonists; when amyl-TMA was allowed to act for a similar period no sensitization occurred. However, when amyl-TMA was used instead of acetylcholine to produce the series of control contractions, octyl-TMA caused the usual increase in sensitivity, so that the effect is clearly not due to inhibition of cholinesterase activity.

These experiments provide strong support for hypotheses 1 and 3. When atropine occupies some, or even most, of the receptors, plenty of others remain; if the concentration of agonist is increased to counter the reduction in the number of receptors available to the agonist, a contraction will occur without the agonist displacing the antagonist. The partial agonists, however, have such a low efficacy that a significant proportion of the receptors must be occupied if a contraction is to be produced; this can only occur when atropine vacates the receptors and the contraction is consequently slow. When the contraction is established most of the receptors will have been freed from atropine, so that when the partial agonist is removed acetylcholine produces nearly its usual effect until the atropine re-establishes itself.

In the experiment shown in Figs. 6 and 7 the presence of atropine only reduced the sensitivity of the ileum to about a quarter, so that the atropine was occupying about 75% of the receptors; the free 25% allowed the partial agonists to produce a rapid initial contraction which then increased slowly as the atropine left the remaining receptors. When octyl-TMA was left in the bath only a little longer than was needed for the rapid phase of the contraction to occur, there was little sensitization; but when the same, or even a smaller, concentration was left in longer the sensitization was more marked (Fig. 6). The size of the rapid part of the contraction due to the 25% free receptors

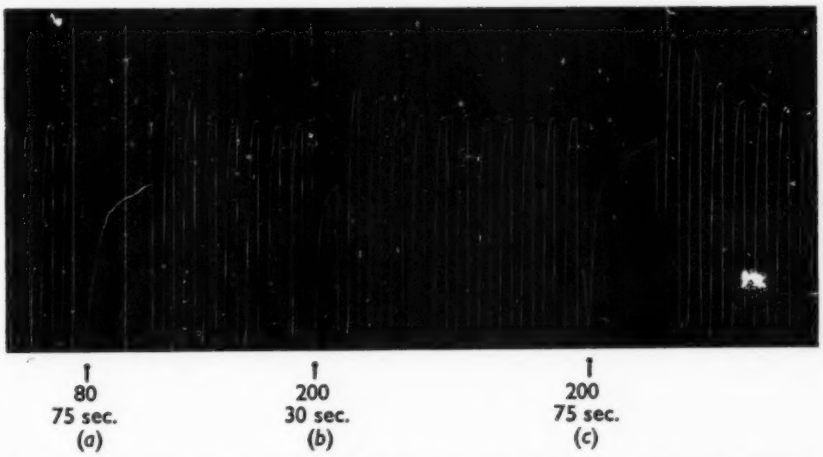

FIG. 6.—Guinea-pig ileum, conditions as in Fig. 2. Atropine sulphate 1 ng./ml. present throughout; regular contractions produced by ACh 20 ng./ml. Octyl-TMA was added at the arrows to give the concentrations shown $\times 10^{-6}$M, and left in the bath for the time stated.

FIG. 7.—Same experiment as in Fig. 6. Atropine present throughout. Alkyl-TMA salts were added at the arrows to give concentrations shown $\times 10^{-6}$M. These concentrations acted for 105 sec.

depended, as would be expected, on the efficacy of the partial agonist ; it was smaller with octyl-TMA than with heptyl-TMA ; with nonyl-TMA it was smaller still (Fig. 7).

The partial agonists with a longer chain did not show the de-atropinization effect so clearly: when used in the concentrations necessary to displace atropine they persisted in the tissue after washing and continued to occupy receptors. The sensitization then appeared as the partial agonist diffused out, and disappeared as the atropine re-established itself. This effect is just apparent with the higher concentration of nonyl-TMA in Fig. 7.

DISCUSSION

So far as the main purpose of this paper is concerned, the evidence about concentration-effect relations is only of secondary interest, and will not be discussed further, except to repeat that there is little experimental justification for the idea that the equation $KA = \frac{y}{1-y}$ expresses a general relation between drug concentration and tissue response ; and that consequently it is unnecessary to assume that the percentage of receptors occupied is equal to the percentage response. The view that such a relation does hold has never been explicitly propounded, but was implied in Clark's discussion of the mode of action of drugs. When clearly stated, the improbability of this relation becomes apparent ; but, since the action of drugs is usually discussed within the framework of Clark's ideas, its widespread acceptance must be assumed. A clear statement of this view occurs in the paper by Raventós (1937b) in which, while discussing the fact that acetylcholine and methyl-TMA have additive inhibitory actions on the frog heart, he says: "If the two drugs occupy a common receptor then the full additive effect must mean that the gross difference in their activity is due to a difference in the ease with which they combine with the tissue. If the difference depended on the amount of drug required to be combined with the tissue in order to produce an effect, then the presence of the weaker drug would tend to antagonize the action of the stronger drug, since the weaker drug would reduce the number of free receptors available for the stronger drug without itself producing much action. . . . A number of the quaternary ammonium salts . . . were tested . . . and an additive effect was found in all such cases between these salts and acetylcholine." The difficulty of allowing different efficacies at once disappears if it is assumed that the effects are produced when only a very small proportion of the receptors are occupied. Only with compounds of very low efficacy would an appreciable proportion of the receptors be occupied and the effect postulated by Raventós appear. It would seem that this does in fact occur with four of the alkyl-TMA salts when tested on guinea-pig ileum.

The surprising observation that the partial agonists oppose the action of atropine, as well as the action of true agonists, strongly supports the idea that the partial agonists, unlike normal agonists, occupy most of the receptors when producing a contraction. In the experiment shown in Fig. 7 atropine reduced the sensitivity to acetylcholine to about one quarter, and was therefore occupying about 75% of the receptors ; the percentage occupied by the alkyl-TMA salts has been calculated from equation 1 using the affinity constants shown in Table V (see below). The results, shown in Table IV, are clearly of the right order to explain the de-atropinization seen in Fig. 7 and illustrate the difference between amyl-TMA and the partial agonists.

This displacement of atropine by the partial agonists would appear to be the effect that Clark (1937b) could not find with acetylcholine and

TABLE IV

CALCULATED PERCENTAGE OF RECEPTORS OCCUPIED BY THE COMPOUNDS TESTED IN THE EXPERIMENT SHOWN IN FIG. 7

Drug and Concentration	Alkyl-TMA Acting Alone		Alkyl-TMA Acting with Atropine		
	% of Receptors		% of Receptors		
	Occupied by Alkyl-TMA	Free	Occupied by Alkyl-TMA	Occupied by Atropine	Free
Atropine ...	—	—	—	75	25
Heptyl-TMA 8×10^{-4}M	96·6	3·4	88·1	8·9	2·9
Octyl-TMA 4×10^{-4}M..	96·0	4·0	86·6	10·1	3·3
Nonyl-TMA 8×10^{-5}M	90·0	10·0	69·3	23·0	7·7
Nonyl-TMA $3·2\times10^{-4}$M	97·3	2·7	90·0	7·5	2·5
Amyl-TMA 4×10^{-3}M..	3·3	96·7	0·8	74·4	24·8

atropine ; he regarded the absence of this effect as evidence against the idea of competitive antagonism. If there is a large excess of receptors then acetylcholine can produce its effect—even though most of the receptors are occupied by atropine—without actually displacing the antagonist from the receptors. Since fewer receptors are available the concentration of acetylcholine has to be increased in order that the same number shall be occupied. Only with very high concentrations of atropine would the production of an effect by acetylcholine require the displacement of atropine—and then only from a very small fraction of the total number of receptors.

The idea that the activity of acetylcholine-like drugs is due to two separate properties—affinity for the receptors, and efficacy—thus seems to explain most of the observed facts ; and, assuming its correctness, it becomes interesting to attempt to separate the contributions of the two factors to the total effect. No attempt has been made to obtain a theoretical relation between the stimulus S and the response R, but the empirical relation can be obtained experimentally since S, like y, is proportional to the concentration when y is small (equation 2a). In Fig. 8 values of S have been plotted against the responses to butyl-TMA from the experiment shown in Fig. 1. From equation 2, and Fig. 8, a set of log-concentration-effect curves for a series of hypothetical compounds with the same affinity, $K=10^3$, but with efficacies varying from 1,000 to 0.5 have been calculated, and are plotted in

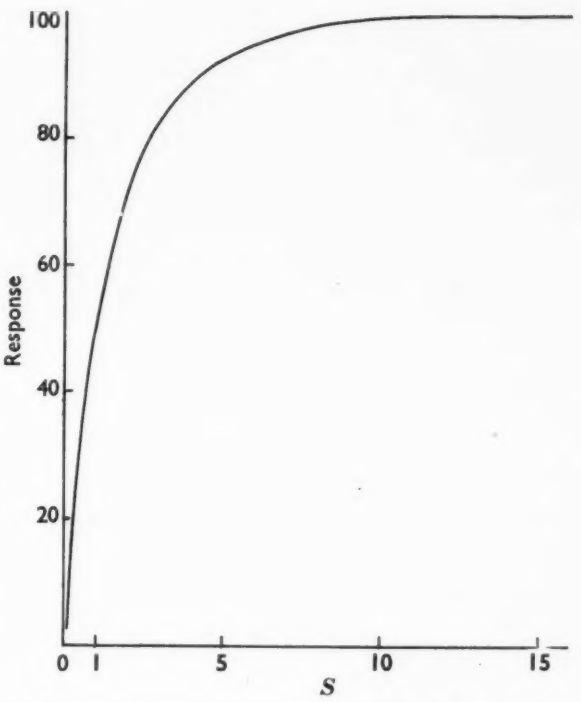

FIG. 8.—Curve relating the response R to the stimulus S ($=ey$) based on the log-concentration-effect curve of butyl-TMA in Fig. 1. Here the effect is plotted against concentration, but the concentration scale is replaced by the arbitrary scale of S. That is, it is assumed that y—the percentage of receptors occupied by butyl-TMA—is small so that y and consequently S are both proportional to concentration.

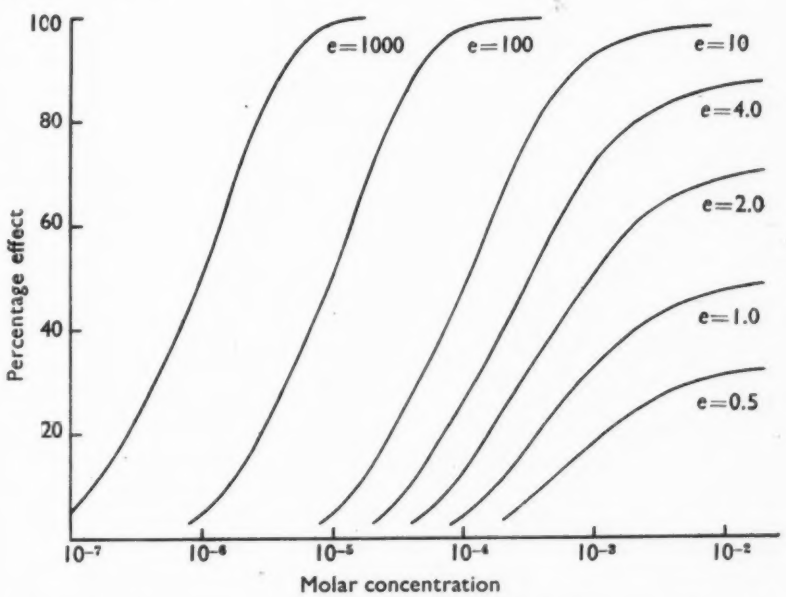

FIG. 9.—Set of log-concentration-effect curves for hypothetical substances. All have the same affinity constant, $K=10^3$, and the efficacy varies as shown from 1,000 to 0.5.

Fig. 9, which shows how the maximal effects produced by partial agonists vary with their efficacy. The ratio of the efficacies of two partial agonists is the same as the ratio of the two concentrations of a true agonist which give contractions equal to the two partial agonist maxima. In systems where the log-concentration-effect curves for true agonists have low slopes, compounds with a wider range of efficacies will come into the class of partial agonists. In these respects " efficacy " differs from " intrinsic activity " which, as defined by Ariëns and Groot, is directly proportional to the size of the maximal response. It is evident that the shape of the curves in Fig. 9 varies little when e is greater than 10, and the curve shown for $e=100$ is practically identical in slope and position with the curve which would result for $K=2\times10^2$ and $e=5\times10^2$, or for any other pair of values such that $Ke=10^5$. Only in special circumstances, therefore, can the separate contributions of K and e be determined.

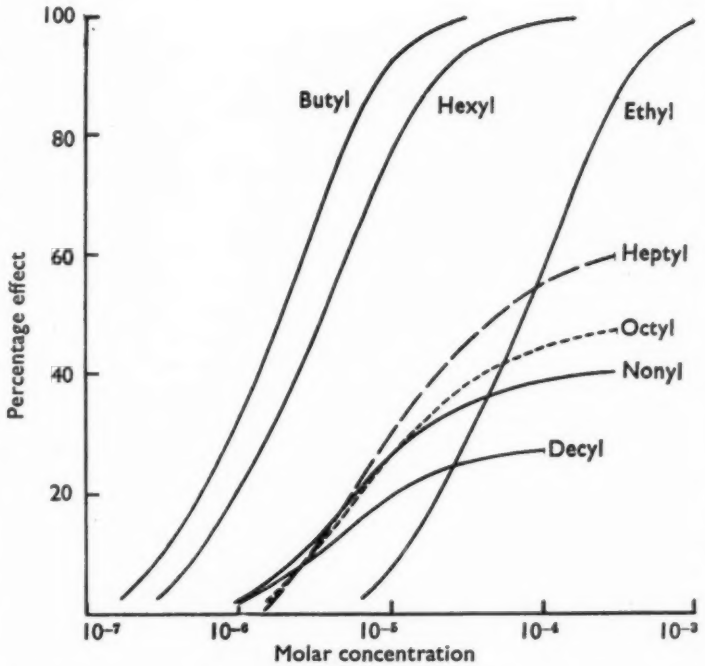

FIG. 10.—Log-concentration-effect curves for the alkyl TMA ions reconstructed from the constants in Table V, using equation 4 and the curve relating R and S shown in Fig. 8. The curve in Fig. 8 is the only link between these curves and the experiment on which Fig. 1 is based. Compare Fig. 10 with Fig. 1.

The extrapolation in Fig. 4 is such a special circumstance, and it provides the basis for estimates of K and e which, though possibly not very accurate, are of considerable interest. The values for the efficacies of the agonists have been calculated from equation 2 using the values of K read from the curved line in Fig. 4 and the concentrations for a 50% response from Table II (so that $S=1$). The efficacies of the partial agonists were obtained by applying equation 3 to the comparisons with butyl-TMA given by the addition experiments. Each experiment gave a value for the appropriate e. The affinity constants and the efficacies of the compounds in the series are listed in Table V.

Once it is accepted that the affinity of the alkyl-TMA ions for acetylcholine receptors varies in a regular way with chain length, it follows that the sharp peak of activity at amyl-TMA is due to high efficacy; this is apparent in Table V. The estimate of the efficacy of methyl-TMA is very dependent on the accuracy of the extrapolation in Fig. 4 and is consequently liable to error: the value obtained is comparable with that for amyl-TMA, and this suggests that most of the difference in the activity of these ions is due to the difference in affinity. The long-chain members of the series have a high affinity and a low efficacy.

TABLE V

THE EFFICACY AND AFFINITY OF ALKYL-TMA IONS

The affinity constants of heptyl-, octyl-, nonyl-, and decyl-TMA are the mean experimental values; the others are read from the curved line in Fig. 4. The values of efficacy are related to the mean sensitivity of the pieces of ileum used to compare the activity of the first 6 members of the series

Alkyl Group	Me	Et	Pr	Bu	Am	Hex	Hep	Oct	Non	Dec
Efficacy	94	31	4·3	200	200	21	2·2	1·4	1·0	0·6
Affinity $\times10^{-3}$	0·24	0·63	1·6	3·8	8·5	19·0	41	63	110	190

These estimates of the affinity constants and of the efficacies of the alkyl TMA salts enable the concentration-effect curves to be reconstructed for comparison with the experimental curves shown in Fig. 1. Since the positions of the curves, and the size of the response to the partial agonists, depend on the sensitivity of the individual piece of ileum, it is necessary to adjust the values of efficacy to the individual case. The ileum on which Fig. 1 is based required 2×10^{-6}M-butyl-TMA for a 50% response, compared with the mean value of 1.33×10^{-6}M in Table II; the values of efficacy appropriate to Fig. 1 are consequently one-third less than those in Table V. From these adjusted efficacies, and the affinity constants in Table V, the curves shown in Fig. 10 have been calculated, using equation 2 to give S, and Fig. 8 to give R. Fig. 10 is a fairly good reproduction of Fig. 1, indicating that the various assumptions which

have been made are not grossly at variance with the experimental evidence.

Action of Antagonists

It is interesting that some antagonists are effective in appreciably lower concentrations than the corresponding agonists (e.g., atropine and acetylcholine; mepyramine and histamine). It follows that such antagonists have higher affinities than the agonists. This difference becomes more marked if we now accept that the agonists cause appreciable responses when very few receptors are involved; and it may be that the incompatibility of high affinity and high efficacy is general, and is not particular to the alkyl-TMA ions.

If the new ideas expressed in this paper—particularly hypothesis 1—are accepted some modification of ideas about drug antagonism will be involved. It is usual to postulate two types of antagonism—competitive and non-competitive—the former being more usual. In competitive antagonism, agonist and antagonist, simultaneously present in solution, are each considered to compete for receptors to the exclusion of the other; and the response is determined by the concentrations of the two drugs and their relative affinity constants. Gaddum's equation (1937) relating these factors is derived from a consideration of the mass laws; its relevance is not affected by hypothesis 1, provided it is realized that the equation gives information about receptors and not about responses. Perhaps the most important point about the type of antagonism usually called competitive is that it is possible to obtain maximum responses with sufficient agonist, and that the slope of the log-concentration-effect curve is not changed in the presence of the antagonist. If, however, considerably more receptors exist than are needed to produce a maximum response, similar results will be possible with antagonists which block receptors irreversibly, or block at some site beyond the receptor but before the number of receptor paths has been appreciably reduced. Such antagonism would obey Gaddum's equation until sufficient paths were blocked to reduce the number of functioning receptors almost to the minimum required for the agonist to produce a maximum response.

There is evidence that the haloalkylamine type of compound blocks several kinds of receptors irreversibly—that is, the response is not restored when the antagonist is removed from the environment (Nickerson, 1949a; Furchgott, 1954), but the blocking action has nevertheless been supposed to be competitive at first, and to become non-competitive only after more prolonged action. This has been discussed by Nickerson (1949b). The implication of a change in mode of action is avoided by the concept of spare receptors. As long as sufficient receptors remain free the antagonism is "surmountable": it is only necessary to increase the concentration of agonist in the inverse proportion to which the available receptors have been reduced. When, however, the number of receptors is irreversibly reduced below the minimum required, maximum responses become impossible; eventually all the receptors are blocked and no contraction can be obtained: the antagonism is "unsurmountable." The two phases of the action are thus seen as stages in a single continuous process in which an increasing proportion of receptors are irreversibly blocked.

This issue is confused because most experiments have involved injecting doses into animals; in such circumstances it is not always clear how long the drug remains in circulation and reversible competition remains possible. This difficulty is avoided in experiments with isolated tissue. Experiments (which will be reported in a subsequent paper) with SY-28 and histamine on guinea-pig ileum show that considerable irreversible block can be associated with a normal slope for the log-concentration-effect curve and a normal maximum contraction. Similar results have been obtained by Nickerson (unpublished). These experiments agree with the above interpretation. It is probable that the haloalkylamine antagonists act directly on the specific receptors (Furchgott, 1954); but it is a considerable modification of the established concept to describe such block, which persists after the disappearance of the antagonist, as competitive antagonism. If hypothesis 1 is correct it is also possible to envisage block occurring away from the receptors producing similar antagonistic effects. Such block whether reversible or not might appear to follow Gaddum's equation over a limited range, but would clearly not be competitive. The equation is strictly applicable only in equilibrium conditions. Equilibrium between receptors and an irreversible drug is only achieved when all the receptors are blocked: it is only the rate at which this condition is approached that is determined by the concentration of drug. Gaddum's equation, and some other expressions of the mass law, have nevertheless been applied in such situations by allowing an arbitrary time of action; sometimes doses injected into animals have been used in place of concentrations in the equation. Used in such ways the equation can carry little theoretical implication and, even when applied to an appropriate experiment, cannot prove that a pharmacological antagonism is competitive. It is conse-

quently preferable to use the purely descriptive terms "surmountable" and "unsurmountable" (Gaddum, Hameed, Hathaway, and Stephens, 1955) to distinguish between the two commonly observed situations. Whether an effect is competitive or not will probably have to be decided on quite different evidence.

Antagonism which is surmountable but irreversible is difficult to explain without accepting hypothesis 1.

Structure-Action Relations

In considering the structural features of a molecule which determine its potency for some specific action, authors, working within the framework of Clark's ideas, have usually considered that these features exert their effects only by modifying the affinity of the molecule for the appropriate receptors. Only occasionally has some suggestion carried an implication of the concept of what has here been termed efficacy. The clear recognition of two separate entities "affinity" and "efficacy," both of which might be altered by any one structural change, should clarify the consideration of many structure-action problems. One example may be mentioned. Holton and Ing (1949) showed a progressive and sharp decline of specific activity as the methyl groups on the nitrogen atom of acetylcholine were successively replaced by ethyl groups. The authors discuss this decline solely in terms of the structural requirements for fitting the acetylcholine receptor. In the closely analogous compounds prepared by Ford Moore and Ing (1945) which block the actions of acetylcholine (the efficacy is reduced to zero by a benzilyl group at the other end of the molecule), the effect of progressively replacing the methyl groups on the nitrogen atom by ethyl groups is not nearly so drastic. The first ethyl group indeed increases the blocking activity and the second and third reduce activity only slightly. Since the activity of blocking compounds must be considered solely in terms of affinity for the receptors, it would seem safe to assume that the trimethylammonium group confers high efficacy rather than high affinity on acetylcholine.

Throughout this paper, receptors have been discussed very nearly as if they existed in isolation. This has been deliberate. What a drug combines with to produce its effect is a subject for speculation. It is attractive to think of a part of some enzyme system at which a stimulant drug becomes a prosthetic group. This must have occurred to most people with interests in this field and has been expressed at least once (Welsh, 1948).

The approach to study of the action of drugs used in this paper is not universally popular among pharmacologists; some, indeed, despise discussion in terms of receptors, so that it is perhaps worth while to try to state my position clearly. The only basic assumption involved when discussing drug action in terms of receptors is that the drugs combine in some way with the tissue they affect. In order to develop a system of thought about drug action it is necessary, in the absence of intimate knowledge, to make additional assumptions. It is important, however, that such assumptions should be made explicitly so that they may be tested where possible; this, unfortunately, is not often done, with the result that mutually conflicting assumptions abound. In this paper one such additional assumption, which is widely—but apparently unconsciously—held, has been challenged, and several other assumptions have been made. Implicit and erroneous assumptions may be concealed in the ideas which have been expressed in this paper. If so, confusion may result and the conclusions drawn may be wrong. But, if the basic assumption is correct, it will be profitable to continue the argument.

Summary

1. The inadequacy of the experimental evidence in favour of the theory that the mass law relation between drug concentration and tissue receptors accounts for the slope of concentration-effect curves is pointed out.

2. A new theory is proposed which avoids the assumption that the percentage effect is equal to the percentage of receptors occupied by a drug. This theory allows a new factor, called efficacy, to be postulated which, together with affinity for the receptors, determines the potency of an agonist drug. This concept removes the absolute distinction between agonist and antagonist drugs, and the existence of an intermediate class of partial agonists is recognized.

3. Experimental evidence for this theory was obtained from an examination of the actions and interactions of the homologous series of alkyl trimethylammonium ions and atropine. Four members of the series acted as partial agonists.

4. The assumption that the percentage response is equal to the percentage of receptors occupied is hidden in much current discussion of drug action. If this assumption is wrong, as is now suggested, the conclusions to be drawn from a wide range of experimental observations will be modified. The concept of competitive antagonism, and ideas

about the relation of chemical structure to pharmacological activity, are discussed.

The experimental work was possible only because Dr. R. B. Barlow undertook the synthesis of the alkyl trimethylammonium salts. I am very grateful.

Some of the material in this paper was discussed at meetings of the British Pharmacological Society in July, 1952, and January, 1954, and at the XIX International Physiological Congress (Stephenson, 1953).

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THE ACTION OF FLUOTHANE*—A NEW VOLATILE ANAESTHETIC

BY

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WITH AN APPENDIX BY R. R. GOODALL

(RECEIVED MAY 31, 1956)

During the search for non-explosive inhalation anaesthetics some authors have examined large numbers of fluoro derivatives of hydrocarbons and ethers. Robbins (1946) found that some fluorohydrocarbons had anaesthetic properties with therapeutic ratios greater than those of ether and chloroform. These compounds, however, produced intense hypotension, particularly in deep anaesthesia, with abnormal cardiac rhythms and sometimes with ventricular fibrillation. More recently Lu, Ling and Krantz (1953) examined some fluorinated hydrocarbons and ethers. Their most promising compound, trifluoroethylvinyl ether (Fluoromar), had, in monkeys, an activity similar to that of ether, but its therapeutic ratio was slightly larger than that of ether. Although this compound seems to be fairly promising, it is explosive when mixed with oxygen in concentrations above 3.0% (Krantz, Carr, Lu, and Bell, 1953). A short clinical report on Fluoromar was published by Orth and Dornette in 1955.

A series of fluorinated hydrocarbons was synthesized by Dr. C. W. Suckling of the General Chemicals Division of I.C.I. and examined in these laboratories (Raventós and Suckling, unpublished). Among them $CF_3CHClBr$ (Fluothane) was found to be a non-inflammable volatile liquid with outstanding anaesthetic properties. The results of experiments on its anaesthetic action and on some of its pharmacological effects are summarized in this article; for the sake of convenience, it is referred to by its trade name.

Physical Characteristics of Fluothane

The physical data on fluothane have been determined by Dr. Pryce, of these laboratories, and by the Physics Section of the Research Laboratories of the General Chemicals Division of Imperial Chemical Industries Limited, Widnes. These data are compared with those of some other anaesthetics in Table I. Fluothane is a liquid with an S.G. of 1.86 at 20° C. and its boiling point is 50.2° C. at 760 mm. Hg. Its vapour pressure at 20° C. is 243 mm. Hg. It has a characteristic but not unpleasant odour which is difficult to define. Like most heavily fluorinated hydrocarbons, fluothane is not inflammable and its vapours mixed with O_2 in proportions from 0.5% to 50% (v/v) are not explosive. Fluothane decomposes slowly with the formation of volatile acids when exposed to light; it is stable, however, if stored in amber-coloured bottles. Thymol in the proportion of 0.01% w/w added to fluothane stabilizes the anaesthetic to the action of light.

Fluothane is stable when in contact with soda lime. When its vapours, mixed with 5% CO_2 + 95% O_2 saturated with water, were recirculated for 2 hr. in a closed system containing soda lime kept at 50° C., taking care to replace the CO_2 absorbed by the soda lime by further additions of this gas, there was only 0.02% decomposition as indicated by the increase in the halide of the soda lime (Suckling, personal communication). Its decomposition has been also studied in experiments on anaesthetized dogs using a close-circuit method. Samples of the vapour mixture were taken from the rebreathing bag at the beginning and after 1, 2, and 3 hr. of anaesthesia. The mass spectra of these samples were not different from that of pure fluothane and the halide content of a sample of the soda lime of the canister taken at the end of the experiment was the same as that of a control sample. These two sets of experiments show that fluothane is stable in contact with soda lime and that it is possible to use it in close-circuit methods of anaesthesia without the formation of toxic decomposition products.

METHODS

Vapour mixtures of fluothane of known concentrations were prepared using a modified Kochmann (1912) apparatus. It can be used for the study of the actions of any volatile liquid.

For the experiments with mice and rats the apparatus shown in Fig. 1 was devised. It consists

* Patent protection pending; registered trade mark of Imperial Chemical (Pharmaceuticals) Ltd.

TABLE I
PHYSICAL CHARACTERISTICS OF SOME INHALATION ANAESTHETICS

	Mol. Wt.	Boiling Point (°C.)	Specific Gravity		Solubility in 100 Parts Water	Oil/ Water Solubility	Oil/ Blood Solubility	Inflammability Limits (%)		Vapour Tension at 20°C. (mm. Hg)
			Gas or Vapour (Air=1)	Liquid (Water =1)				Air	O_2	
Chloroform	119·39	61·26	4·12	1·49	0·822	100		—	—	160·5
Cyclopropane	42·08	−34·4	1·42	—	33 ml.	34·3	15·3	2·4–10·3	2·45	
Ether	74·12	34·6	2·6	0·713	7·5	15·46 3·2	15·08 3·3	1·85–36·5	2·10–82·0	439
Divinyl ether	70·09	28·3	2·2	0·774	4·0	41·3		1·7–27·0	1·85–85·0	
Ethyl chloride	64·52	12·2	2·28	0·921	0·574			4·0–14·8		
Ethylene	28·05	−103	0·978	—	25 ml. at 0°C. 10 ml. at 25°C.	14·4	9·3	3·5–15	1·5–85·0	
Nitrous oxide	44·2	−181·0	1·527	1·226	150 ml.	3·2	3·0	—	—	49·4
Fluoromar .. .. $CF_3—CH_2—O—CH=CH_2$	126	42·7	—	1·13	0·4	94		3·0		
$CF_3—CHClBr$ .. .. Fluothane	197·39	50·2	—	1·86	0·345	330	—	Not inflammable in O_2 0·5 to 50·0		243

of a vaporization chamber, an exposure chamber and a recovery tower.

The vaporization chamber (1) is a test-tube (1½ by 8 in.) kept in a thermostatically controlled bath at the boiling point of fluothane. The end is closed with a rubber bung through which pass two ¼ in. glass tubes and one capillary. One of the larger tubes has a glass jacket around it and almost reaches the bottom of the chamber. Air or O_2 is introduced by this tube at a constant rate measured by a rotameter. The volatile liquid is forced into the chamber through the capillary tube and falls in drops on the glass jacket. The liquid spreads over the jacket and volatilizes before it reaches the bottom of the chamber; the vapour produced is mixed with air or O_2 and passes out by the second ¼ in. tube.

The rate of introduction of fluothane into the chamber is regulated as follows. The volatile liquid is contained in a cylindrical glass vessel (2) connected to two capillary tubes. One protrudes about ½ in. into the upper quarter of the vessel and is fixed horizontally within it (3); the other capillary is fixed vertically, and at its upper end has a three-way tap. The vertical arm of the tap is fitted with a small funnel, which is used for filling the vessel with the volatile liquid; the horizontal arm (4) is the outlet of the vessel. At the bottom of the vessel there is another tap for emptying it. Mercury is introduced into the vessel through the capillary tube (3) at a constant rate by means of a continuous injection apparatus (5), and displaces the volatile liquid into the vaporization chamber.

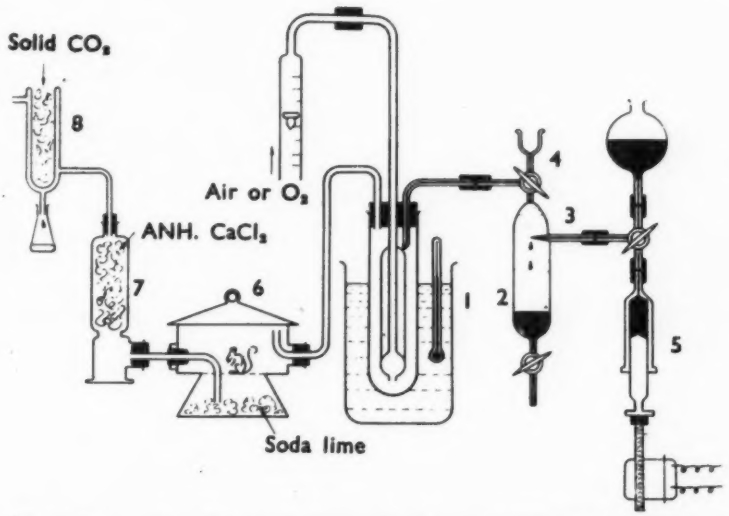


FIG. 1.—Diagram (not to scale) of apparatus for exposing rats and mice to known concentrations of fluothane—see text for description.

Rats and mice are exposed to the vapour on a wire mesh in the upper part of a desiccator of about 10 l. capacity (6). The lower part contains soda lime. The outlet of the exposure chamber is connected to an anhydrous $CaCl_2$ tower (7), where the moisture of the vapour mixture is removed. Finally, the mixture passes into a solid CO_2 condenser (8), where the anaesthetic is condensed. The recovered material can be used again after distillation.

For experiments on rabbits, dogs, cats, and monkeys it was necessary to modify this apparatus (Fig. 2). The outlet of the vaporization chamber is connected to a series of polythene bags

where the vapour mixtures are collected (1). The mouths of these bags are tied around large rubber bungs fitted with inlet and outlet tubes. The outlet tube is connected to a wide-bore T-piece; one of its branches can be closed or left open to the air, and the other branch, which contains a short glass tube acting as a light gas resistance, is connected to the damping chamber of a modified Gaddum's (1941) apparatus for recording respiratory movements (3). Its outlet is connected by a rubber tube to the inspiration side of a set of unidirectional respiratory valves (4). The expiratory valve is connected to the recovery system.

The tubes and connexions in this apparatus must have an internal diameter sufficiently large not to produce excessive resistance to the flow of the gas mixtures. For experiments on dogs it was found that the best size was 1 in., and that polythene bags of approximately 50 l. capacity were sufficiently large for most experiments. The damping chamber was a 3 l. round-bottomed flask fitted with a rubber balloon of 4 in. diameter. For experiments on cats, rabbits, and monkeys the diameters of the tubes and sizes of the bags were reduced to one quarter.

The gas laws were used to calculate the concentrations of the vapour mixtures. The volume of vapour produced is given by the following formula:

$$\frac{V \times S.G.}{M.W.} \times 22.4 = \text{l. vapour/min. at S.T.P.}$$

where M.W. is the molecular weight of the compound, S.G. its specific gravity, and V the volume of liquid vaporized/min. The % concentration of vapour in air or O_2 was calculated in terms of v/v, and is expressed thus unless otherwise stated.

Rats and Mice.—After flushing the exposure chamber with the vapour mixture for at least 5 min., batches of 10 mice or 4 rats were introduced into the chamber as quickly as possible (in not more than 30 sec.). The animals were observed at regular intervals and the incidence of anaesthesia (side position) and death were recorded. The vapour concentrations that anaesthetize (AC50) or kill (LC50) half the animals after 30 min. exposure were calculated. This time of exposure was chosen because it was thought that the concentration of the anaesthetic in the blood would then be virtually in equilibrium with that of the vapour mixture.

Dogs and Cats.—In all, 48 dogs—beagles of 10–14 kg. body weight—were used. As a rule, they were premedicated with 2–3 mg./kg. morphine sulphate and 0.05 mg./kg. atropine sulphate, both given subcutaneously about 1 hr. before the experiments. The inguinal region was anaesthetized with 2% procaine hydrochloride. The femoral artery was connected to a mercury manometer. An airtight mask was fixed on the dog's face and connected to the inhalation apparatus, with the mixture bags by-passed. Control records of the respiration and blood pressure were taken for about 5 min. before starting inhalation of the anaesthetic mixture.

In some dogs, anaesthesia was induced with thialbarbitone, 40–50 mg./kg. i.v. Control tracings of the blood pressure and respiration were taken until the dog showed the first signs of recovery from the effects of thialbarbitone. As soon as these appeared, inhalation of fluothane in concentrations between 2% and 4% was begun until full surgical anaesthesia was obtained, usually in about 3–8 min. The trachea was then intubated, using an endotracheal tube with an inflatable cuff, and the inhalation of the anaesthetic in high concentrations was continued for a few min. more, after which lower concentrations could be used. No dissection was carried out in unpremedicated dogs until they were fully anaesthetized with fluothane.

Other observations were carried out on dogs and cats anaesthetized with chloralose, 60–80 mg./kg. i.v. A total of 19 dogs and 17 cats were used in these experiments.

The cardiac output of two dogs, premedicated with morphine, was measured before and during anaesthesia by the Evans-blue dilution method. The cardiac output and cardiac index were calculated by the method of Lewis (1953).

The concentration of fluothane in the blood of dogs was estimated during anaesthesia by the method of Goodall described in the appendix.

Monkeys.—Eight rhesus monkeys were anaesthetized with thialbarbitone (30–40 mg./kg. i.v., or more if necessary). Sometimes a mask was placed on the face and connected to

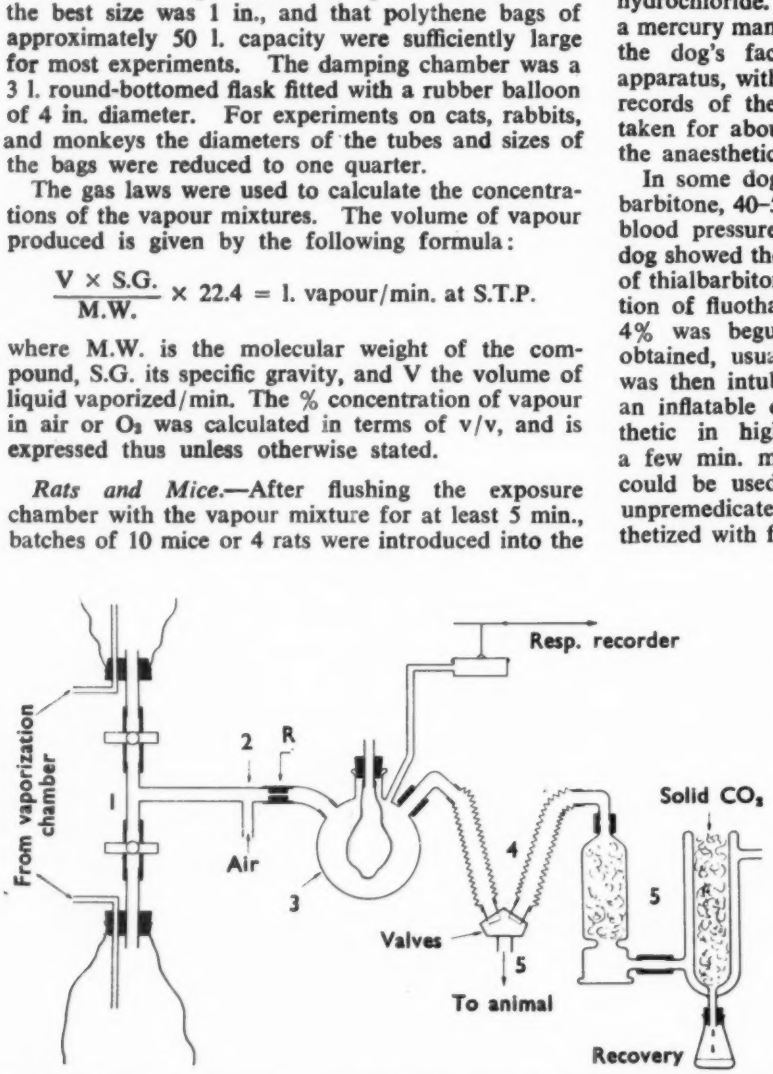


FIG. 2.—Diagram of apparatus for exposing larger animals to known concentrations of fluothane—see text for description.

the inhalation apparatus. Otherwise an endotracheal tube with an inflatable cuff was inserted after spraying the larynx with a solution of cocaine hydrochloride, while the monkey was still under the influence of thialbarbitone. Inhalation of fluothane was not started until the monkey showed the first signs of recovery. The respiration and the ECG were recorded.

Isolated Tortoise Atrium.—The tortoise *T. groeca* was decapitated, its plastron removed, and strips of both atria containing the sinus were suspended in a 10 ml. bath of Clark's frog Ringer at room temperature. The contractions were recorded on a smoked drum, using a light isotonic lever. The Ringer solutions, saturated with ether, chloroform, and fluothane, were prepared by shaking, for approximately 20 min., an excess of the volatile liquids in Ringer solution and letting them stand until complete separation of the two phases occurred. Saturated solutions of cyclopropane were prepared by bubbling the gas through Ringer solution for at least ½ hr. The final concentration of each anaesthetic in the bath was calculated from its solubility in water, and no correction was made for any change of solubility due to the salts of the Ringer solution. The height of the contraction of the strips was measured before and after the addition of the anaesthetic solutions, and the degree of inhibition produced by the anaesthetic was calculated as a percentage of the normal contraction of the strip.

Hepatic and Renal Function Tests.—The hepatic function of dogs anaesthetized with fluothane was estimated by the bromosulphalein method of Reinhold and Hutchinson (1954), and in rats by the hippuric acid excretion method of Quick (1940).

The renal function of rats and dogs anaesthetized with fluothane was examined by the phenol red excretion test (Hawk, 1954). The water diuresis test was also used. Rats were starved overnight and given 5 ml./100 g. of tepid tap water by mouth; urine was then collected at ½ hourly intervals for 3 hr.

Electro-encephalography.—Records of the EEG of rabbits with electrodes implanted in their skull were taken during consciousness and at different levels of anaesthesia. The electrodes consisted of a $\frac{1}{16}$ in. silver rod embedded in a perspex sleeve. One end of the sleeve was screwed in a trephine hole made in the skull and the other was outside the skin. One end of the silver electrode made contact with the dura and the other could be connected to the recording apparatus.

Histopathology.—Animals anaesthetized with fluothane for different times were allowed to recover and kept under observation for at least 2 days. They were then killed and specimens of their tissues were obtained immediately after death and fixed with formol-saline or with acetic acid-Zenker solutions. The microscopical sections of these specimens, obtained by standard methods, were examined by my colleague, Dr. Paget.

Definition of Surgical Anaesthesia.—The term "surgical anaesthesia" is used in this paper to describe a level of anaesthesia similar to that of Stage III, plane II–III, of Guedel's classification (1951), i.e., anaesthesia with loss of corneal reflex, predominantly abdominal respiration and muscular relaxation.

Results

Mice.—The anaesthesia produced in mice with fluothane is characterized by the rapidity of its onset. In experiments with 1.0 to 2.0% fluothane most mice were anaesthetized in less than 5 min. Recovery from anaesthesia was equally fast, and mice that had been anaesthetized for over 1 hr. recovered fully in 5–6 min. after their removal from the exposure chamber.

The AC50 and LC50 of fluothane and other inhalation anaesthetics are summarized in Table II, which shows that fluothane is about 1.5 times as potent as chloroform and about five times as potent as diethyl ether. Its AC50 is lower than that of all the anaesthetics of Table II with the exception of trichloroethylene which, under the conditions of these experiments, seems excellent. The ratio $\frac{LC50}{AC50}$ of fluothane, 3.3, is about twice that of ether, 1.7, and of chloroform, 1.5.

TABLE II

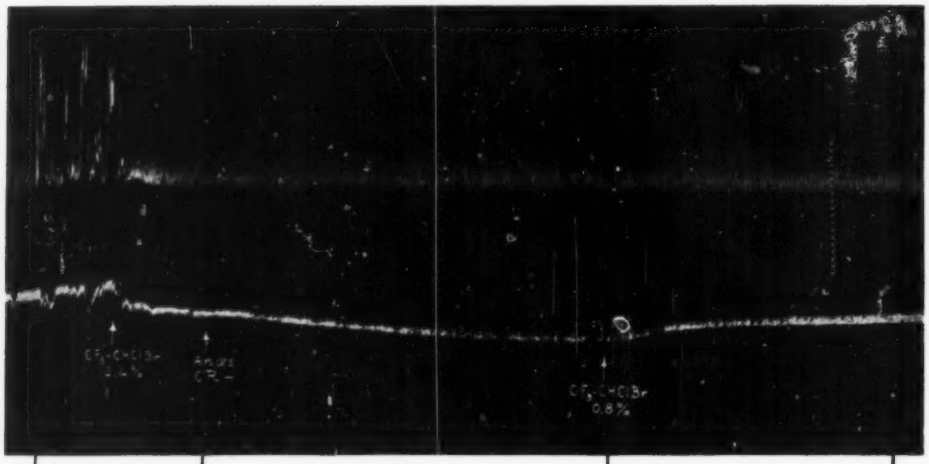
EFFECT OF INHALATION OF ANAESTHETICS ON MICE AFTER 30 MIN. EXPOSURE

Numerals in parentheses indicate 95% confidence limits

Compound	AC50 (Vapour, %)	LC50 (Vapour, %)	Ratio LC50/AC50
Chloroform ..	1·3 (±0·13)	2·0 (±0·1)	1·5 (±0·16)
Diethyl ether	4·3 (±0·215)	7·3 (±0·51)	1·7 (±0·15)
Trichloroethylene ..	0·82 (±0·041)	4·9 (±0·29)	5·0 (±0·35)
Cyclopropane	17·4 (±1·57)	25·8 (±2·06)	1·5 (±0·18)
Ethyl chloride	4·7 (±0·28)	8·2 (±0·74)	1·7 (±0·19)
Fluothane ..	0·86 (±0·069)	2·8 (±0·34)	3·3 (±0·46)

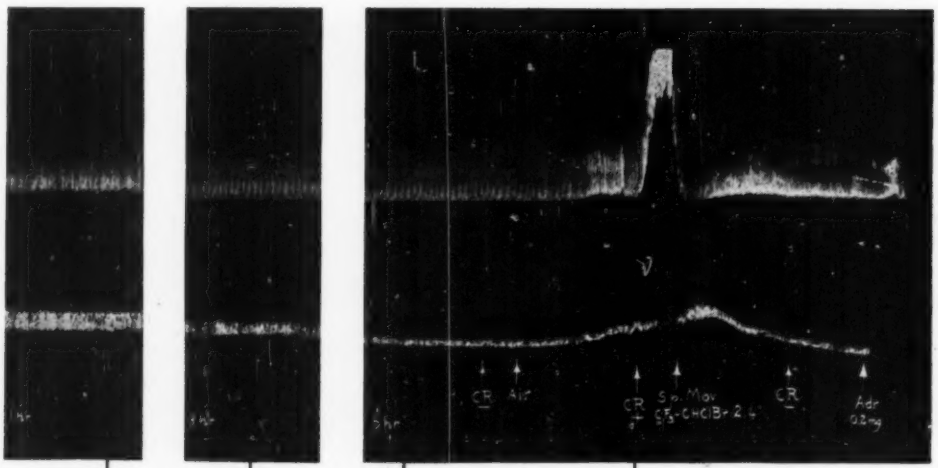
Dogs and Monkeys.—Concentrations of fluothane between 2% and 4% in air or in O_2 were sufficient for induction of anaesthesia, and there was scarcely any excitement. Full surgical anaesthesia was obtained after 2–5 min. whether the dogs were premedicated or not. At this point, both corneal and laryngeal reflexes were abolished, and the trachea could be intubated without provoking coughing (Fig. 3a). As the apparatus used in these experiments had a fairly large dead space, the times of onset of anaesthesia were more or less the same with any concentration of fluothane within the limits mentioned above.

When surgical anaesthesia had been obtained, the concentration of fluothane could be decreased to a minimum which maintained anaesthesia at a steady level for several hours. This concentration



H.rate	130	130	95	110
B.P.	140	130	105	130
R.rate	13	17	14	17
T.vol.	150	125	135	150

FIG. 3a.—Dog 14½ kg. Induction, 30 mg./kg. Na thialbarbitone i.v. In the control period the dog was recovering from the effects of thialbarbitone. At the first arrow, beginning of the inhalation of 2.4% fluothane. Surgical anaesthesia with loss of corneal reflex was observed at the second arrow. Third arrow, change over to maintenance concentration, 0.8% of fluothane. Upper tracing, respiration; middle tracing, blood pressure; and lower tracing, time in 30 sec. Heart rate, blood pressure, respiratory rate, and tidal volume are given below the tracing.



H.rate	120	105	120	125
B.P.	135	130	115	135
Resp.rate	24	22	23	30
Tidal vol.	160	160	160	225

FIG. 3b.—Continuation of Fig. 3a. Records obtained 1, 3, and 5 hr. after the beginning of anaesthesia with fluothane. At 5 hr.: first arrow, negative corneal reflex; second arrow, cessation of anaesthesia; third arrow, positive corneal reflex; fourth arrow, first stage of recovery and spontaneous movements. Inhalation of 2.4% fluothane: fifth arrow, negative corneal reflex; sixth arrow, i.v. injection of 0.2 mg. of adrenaline and ventricular fibrillation.

varied from dog to dog and also depended on whether the dogs had been premedicated. In about 5% of the dogs which had received morphine or thialbarbitone, it was possible to maintain anaesthesia by the inhalation of fluothane in concentrations as low as 0.4–0.5%. More usually, anaesthesia was maintained with 0.8% fluothane (Fig. 3a and 3b). This was not sufficient in unpremedicated dogs, which required about 1.0–1.2% of fluothane for the maintenance of anaesthesia.

In similar experiments on premedicated dogs, it was found that the maintenance concentrations of ether and chloroform were at least 3.0% and 1.5% respectively. If the maintenance concentrations of the anaesthetics are taken as a basis of comparison of their relative potency, fluothane appears to be about twice as potent as chloroform and four times as potent as ether, a result very similar to that obtained in mice.

The concentrations of fluothane needed for the induction and maintenance of anaesthesia in monkeys pretreated with thialbarbitone were approximately the same as those found effective in premedicated dogs. As all the monkeys used in this series were pretreated with thialbarbitone, it is not possible to say whether the concentrations active in the unpremedicated dogs mentioned above would also be active in monkeys under the same conditions.

Recovery from anaesthesia with fluothane was equally fast and free from excitement in all the animal species in which it has been tried. All animals recovered completely in about 10–20 min., even from periods of anaesthesia lasting 5–6 hr., and they were then able to walk normally. The first signs of recovery appeared sooner, as is shown in Fig. 3b, where the corneal reflex became positive in 2 min. and spontaneous movements were observed 5 min. after the cessation of the inhalation of fluothane.

Other Methods of Administration and Surgical Interventions.—Besides the experimental method used in this series, which can be considered as an open-circuit method, some clinical methods of anaesthesia were tried on animals. The open mask method was tried successfully in dogs, cats, and rabbits, but the level of anaesthesia was somewhat difficult to control, and relatively small changes in the concentration of the agent markedly influenced the depth of anaesthesia. As the action of the substance is characterized by rapidity of induction and recovery, it is not surprising that the open mask method of anaesthesia should be difficult to control. Unpremedicated dogs and cats have been anaesthetized by this method without any undue excitement during induction. Recovery was as rapid as after anaesthesia by the experimental method. Fluothane has also been used in close-circuit methods, and dogs have been anaesthetized for as long as 4 hr. using a Boyle's or a Gillies apparatus.

In operations carried out in dogs anaesthetized with fluothane, the muscular relaxation produced was sufficient for abdominal interventions, such as the preparation of stomach pouches. Another striking observation was the scarce capillary bleeding of both skin and muscle incisions. The operative field was therefore free from blood.

Respiration.—Both the amplitude and frequency of respiratory movements were decreased during anaesthesia with fluothane. The reduction in the respiratory rate was, as a rule, more than the reduction in amplitude. In our dogs, weighing 10–14 kg. at the level of surgical anaesthesia, the rate could be as low as 15–25/min. with a tidal volume of about 125–250 ml. (Figs. 3a and 3b).

As with other anaesthetics, the thoracic respiratory movements were abolished earlier than the diaphragmatic so that the latter became predominant as the level of surgical anaesthesia was reached. The inhalation of fluothane in concentrations of 3.0% or lower for 1 hr. did not stop respiration. Higher concentrations stopped respiration in a time inversely proportional to the concentration. In five experiments the inhalation of 3.6% fluothane was continued until respiratory arrest was produced, in an average of 60 min. from the begining of the experiment. In Table III the times given by Guedel (1951) for the production of respiratory arrest with induction concentrations of some anaesthetics are compared with those observed in experiments with different concentrations of fluothane.

When the respiration ceased, the blood pressure was still reasonably high, between 60–80 mm. Hg, and the heart continued beating for about 9 to 10 min. This time-lag between respiratory and

TABLE III

TIMES NEEDED FOR THE PRODUCTION OF RESPIRATORY ARREST WITH INDUCTION CONCENTRATIONS OF SOME ANAESTHETICS

Anaesthetic	Time (min.)	Authority
Ether	10–30	Guedel (1951)
Cyclopropane	½–3	
Ethylene	1–4	
Chloroform	4–12	
Nitrous oxide	1–4	
Ethyl chloride	1–4	
Fluothane 3·0%	No paralysis in 1 hr.	This paper
3·5%	45–60	
,, 4·0%	20–30	

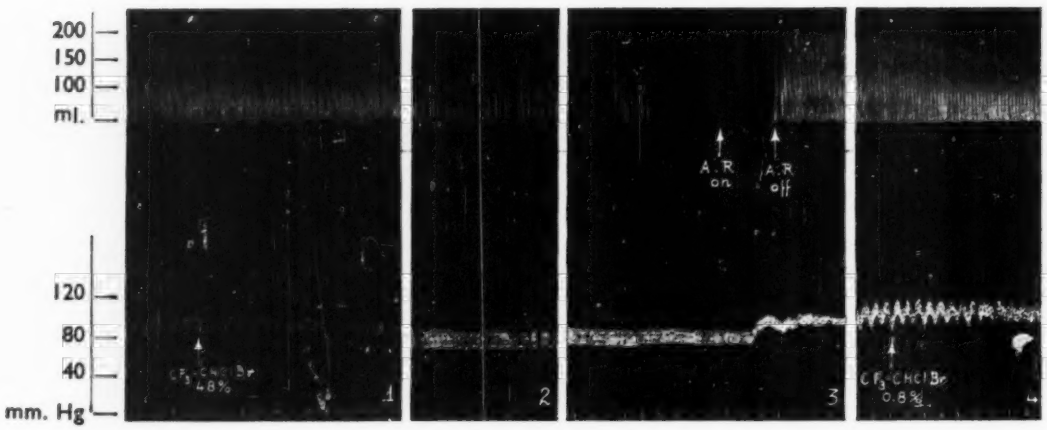


FIG. 4.—Respiratory arrest in a dog after 4 hr. anaesthesia, produced by increasing the concentration of fluothane. The dog inhaled 0.8% fluothane for 4 hr., and at the first arrow the concentration was increased to 4.8%. The inhalation of this concentration was continued for 35 min. until respiratory arrest (3). Artificial respiration (A.R.) and return of spontaneous respiratory movements 3 min. later.

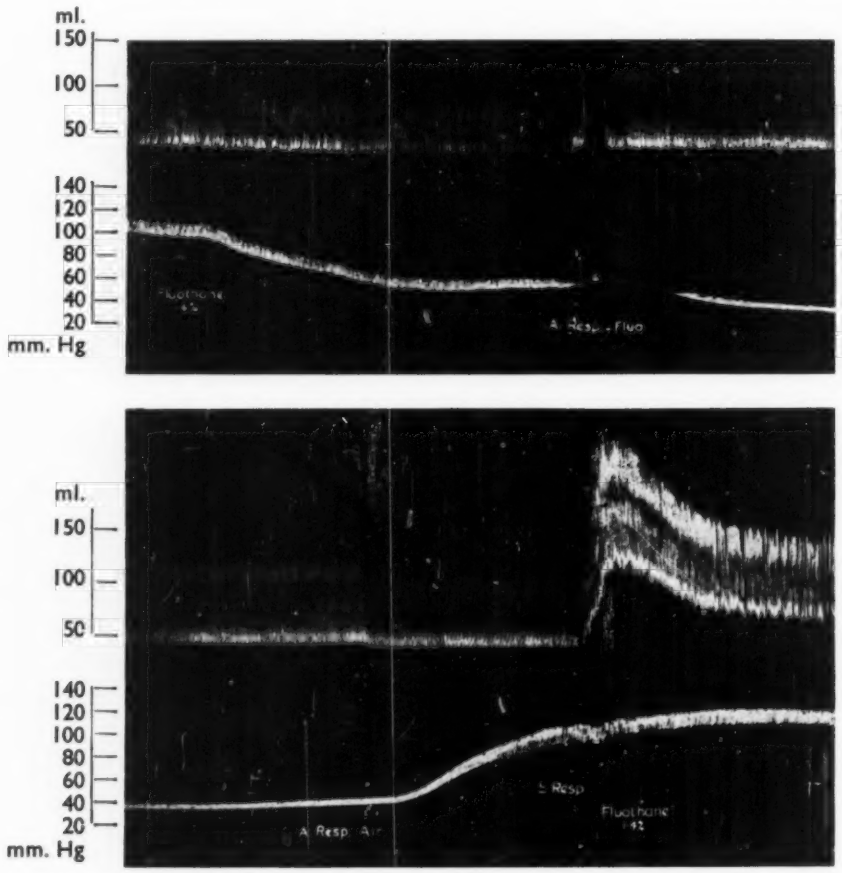


FIG. 5.—Dog 11 kg. Induction, fluothane 4.0%. Maintenance 1.2% fluothane in O_2. Upper record: The dog had inhaled 1.2% fluothane for 1 hr. when the anaesthetic concentration was increased to 4.0%. Respiratory arrest 9 min. later and start of administration of the same anaesthetic mixture by means of an artificial respiration pump (A.Resp.+Fluo). Lower record: After 90 min. of administration of 4.0% fluothane by means of artificial respiration pump. A.Resp.Air, start of artificial respiration with air. S.Resp., spontaneous respiratory movements. Fluothane 1.4% was given at the end of the experiments. In both records the top tracing is the respiratory movement and the lower tracing is the blood pressure. Time, 30 sec. See Fig. 12 for ECG records of this experiment.

cardiac arrest is fairly long and provides plenty of warning when a dangerous level of anaesthesia has been reached.

The respiratory arrest produced by fluothane was easily reversible and normal respiratory movements were usually present after a few minutes of artificial respiration. Even when this arrest was produced deliberately at the end of a long anaesthesia, by increasing the concentration of the anaesthetic, it was found that the apnoea could be reversed by artificial respiration with air or O_2 (Fig. 4). In three experiments the administration of these high concentrations of fluothane was continued for 1½ hr. after respiratory arrest by means of an artificial respiration pump. The apnoea produced in these experiments was reversed with the same ease when artificial respiration was continued with air instead of the anaesthetic mixture (Fig. 5), and the recovery was not appreciably longer than from anaesthesia of long duration where no apnoea was produced.

In other experiments, once the dogs were anaesthetized with fluothane, their respiratory movements were arrested by means of (+)-tubocurarine and the administration of the anaesthetic was continued with a Starling respiration pump. In the experiment of Fig. 6 a first dose of 5 mg. of (+)-tubocurarine was administered and 6 further doses of 2 mg. were given when necessary, i.e., as soon as spontaneous respiratory movements occurred. The first dose of (+)-tubocurarine produced a fall of blood pressure, but once its effects had disappeared the pressure remained at the same level during the experiment, which lasted for 4 hr.

Blood Pressure.—The inhalation of fluothane produced a fall of blood pressure, roughly proportional to the concentration of the vapour. This effect was larger during the induction period when high concentrations of 2.0–4.0% were administered, and reached its maximum about 30–40 min. after the beginning of the inhalation, or 23–35 min. after the production of surgical anaesthesia. The hypotension was relatively small in dogs, in which the pressure fell from 120–140 mm. Hg to 90 mm. Hg; it was larger, however, in cats and rabbits. A comparison of Figs. 3a and 3b, taken from an experiment on a dog, with Figs. 7 and 8 from experiments on cats, shows this difference.

When full surgical anaesthesia had been obtained, the hypotension was not great, and the blood pressure rose as soon as the dogs were put on to the maintenance concentration (0.8% to 1.0%). It remained between 100–130 mm. Hg for the rest of the experiments, provided the concentration of the vapour mixture was kept constant (Figs. 3a and 3b).

Blood pressures between 60–80 mm. Hg were recorded when the respiration was stopped by the

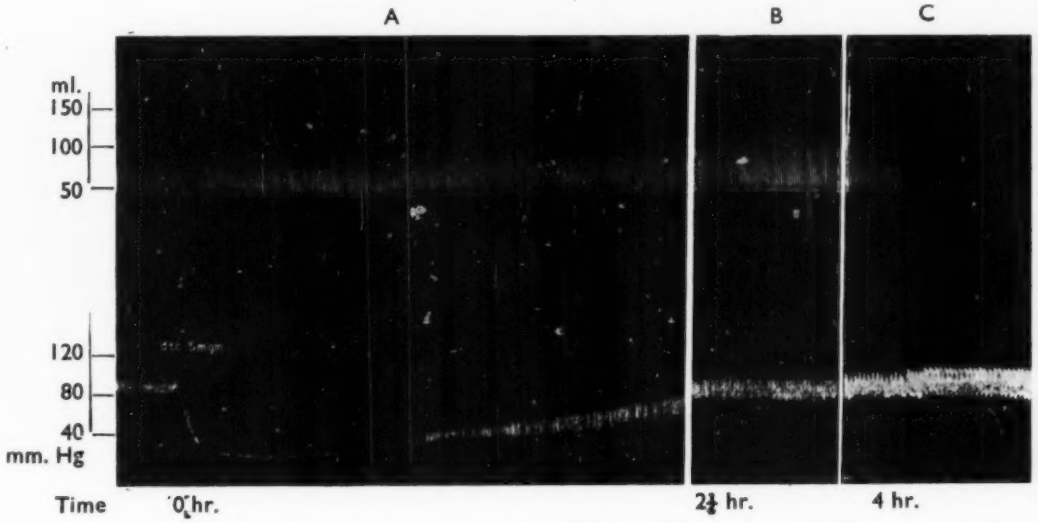


FIG. 6.—Dog 12.5 kg. Premedication, morphine+atropine; induction, fluothane 2.4%; maintenance, fluothane 1.2%. A, 1 hr. after induction. At 0 hr. 5 mg. of (+)-tubocurarine (TC) i.v. and beginning of the inhalation of the anaesthetic mixture (1.2%) by means of an artificial respiration pump. B, 2½ hr. after the first dose of TC. Five further doses of 2 mg. TC were given between A and B. C, 4 hr. after the first dose of TC. The seventh and last dose of 2 mg. TC was given 45 min. before C. Administration of fluothane stopped, artificial respiration and return of spontaneous respiration. Top tracing, respiration; lower tracing, blood pressure. Time, 30 sec. See Fig. 13 for ECG records of this experiment.

inhalation of high concentrations of fluothane, and fell to 40–50 mm. Hg if the administration of these vapour mixtures was continued by means of an artificial respiration pump. However, the blood pressure returned to normal levels soon after starting artificial respiration with air or O_2 (Fig. 5).

The cause of the hypotension produced by fluothane has been studied by several methods. It was found that plethysmographic records of the spleen (3 expt.) and intestine of cats (4 expt.) anaesthetized with chloralose showed an increase in the volume of these organs (Fig. 7). Under the same conditions, the volume of the leg (4 expt.) was not altered and there was a reduction in the volume of the kidney (Fig. 8).

During the inhalation of fluothane in cats and dogs anaesthetized with chloralose, there was a block of conduction in the sympathetic ganglia. This was observed in the following experiments: (*a*) the contraction of the nictitating membrane produced by the preganglionic stimulation of the cervical sympathetic nerve was reduced, (*b*) the hypertension produced by large doses of acetylcholine in atropinized animals was abolished,

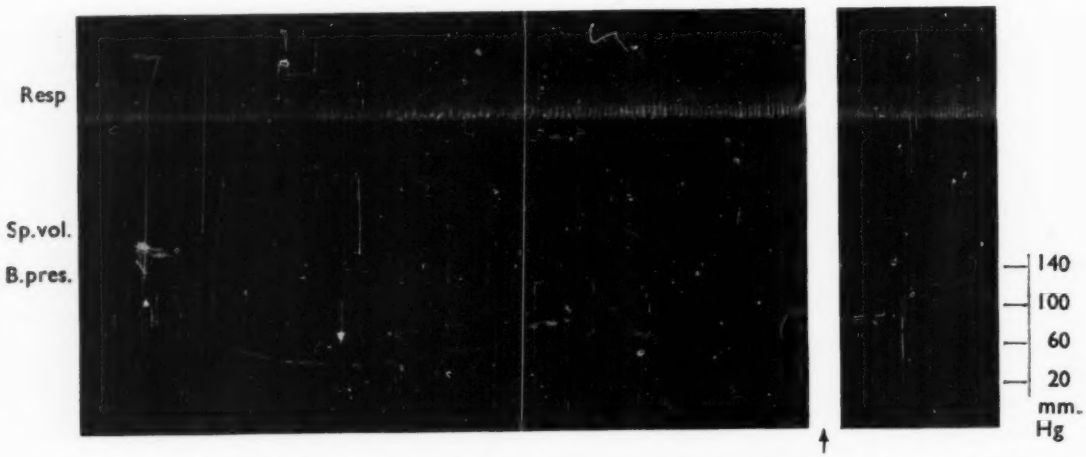


FIG. 7.—Action of fluothane on the spleen volume of a cat anaesthetized with chloralose (70 mg./kg. i.v.). Fluothane (2.4%) was inhaled between the two arrows. Resp., respiration; sp.vol., plethysmographic record of the spleen volume, increase of spleen volume upwards; B.pres., blood pressure. Time, 30 sec.

FIG. 8.—Action of fluothane on the kidney volume of a cat anaesthetized with chloralose (70 mg./kg. i.v.). Fluothane (2.4%) was inhaled between the two arrows. Resp., respiration; K.vol., plethysmographic record of kidney volume, decrease of volume downwards; B.p., blood pressure. Time, 30 sec.

(*c*) the hypertension produced by stimulation of the splanchnic nerve, or (*d*) by occlusion of the carotid arteries was reduced (Figs. 9, 10, and 11). All these effects were reversible and disappeared shortly after inhalation of the anaesthetic had ceased. There is, however, some specificity in the action of fluothane on different ganglia, and from the preliminary results reported here it seems likely that the mesenteric ganglion is more sensitive to its action than the superior cervical ganglion.

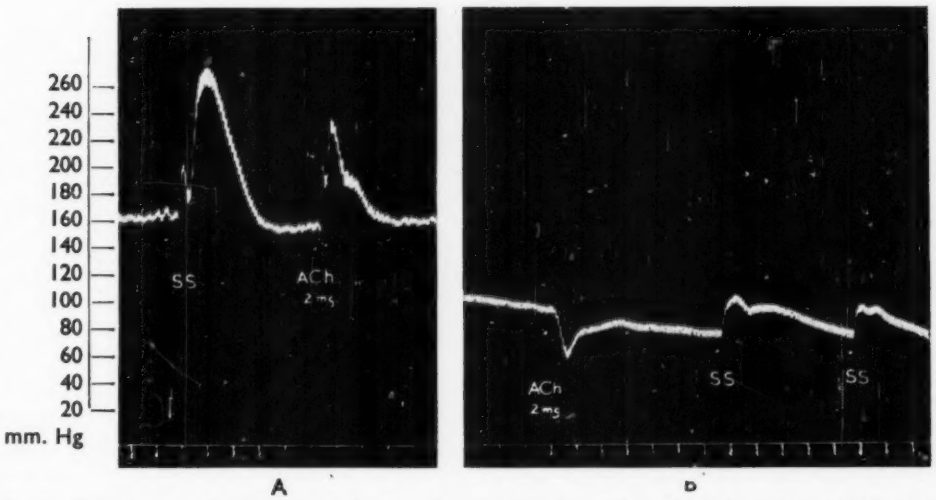


FIG. 9.—Dog 11.1 kg. Chloralose 80 mg./kg. i.v. Atropine 5 mg. i.v. Effects of the stimulation of the splanchnic nerve (s.s.) and of 2 mg. acetylcholine (ACh) on the blood pressure of a dog before (A) and during (B) the inhalation of 2.4% fluothane. Time, 30 sec.

In experiments, such as that of Fig. 9, the inhalation of 2.4% fluothane for 10–15 min. abolished the nicotinic action of large doses of acetylcholine and it produced a considerable decrease of the effects of the stimulation of the splanchnic nerve. On the other hand, this concentration of fluothane produced only between 25–50% decrease in the height of the contraction of the nictitating membrane of 6 cats caused by maximal preganglionic stimulation of the cervical sympathetic nerve (Fig. 11).

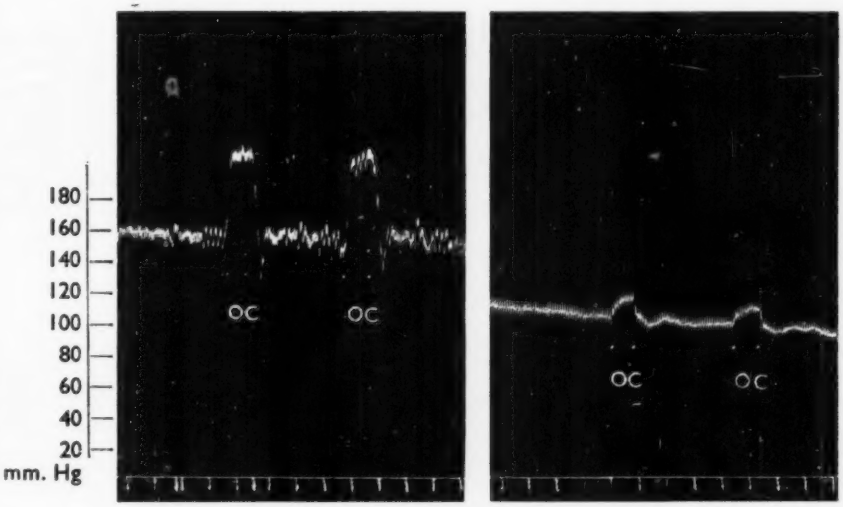


FIG. 10.—Dog 12.3 kg. Chloralose 80 mg./kg. i.v. Effects of the occlusion of the carotids (OC) on the blood pressure of a dog before (A) and during (B) the inhalation of 2.4% fluothane. Time, 30 sec.

These results cannot be due to a reduction of the peripheral action of adrenaline or noradrenaline because the pressor effects of the i.v. administration of small doses of these amines is not modified by the inhalation of the fluothane. The ganglion-blocking properties of other anaesthetics have been described by Larrabee, García Ramos, and Bülbring (1952).

In two dogs premedicated with morphine, no significant difference was found between their cardiac output and cardiac index measured before and during the inhalation of maintenance concentrations of fluothane. Since the cardiac output was not altered during anaesthesia with fluothane it can be assumed that vasodilatation in the splanchnic area, probably due to a sympathetic ganglion block, is the main cause of the hypotension.

Heart Rate and EEG.—A decrease in the heart rate was observed in all animals under the action of fluothane. During the induction period in dogs, a reduction from 130–170 to 80–100 beats/min. was recorded, but the heart rate rose again to about 120–140 beats/min. shortly after the beginning of inhalation of the maintenance con-

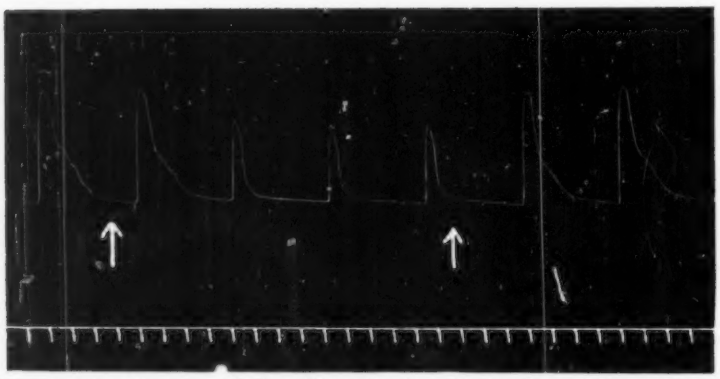


FIG. 11.—Cat 3.2 kg. Chloralose 70 mg./kg. i.v. Contraction of the nictitating membrane produced by supramaximal preganglionic stimulation (20 stim/sec. for 12 sec. every 2 min.). Fluothane (2.4%) was administered between the arrows.

centration ; it remained within these limits as long as the low concentration of fluothane was inhaled (Fig. 3a and 3b).

The electrocardiograms, lead II, of dogs and monkeys anaesthetized with fluothane did not differ from those taken in the same animals during the control periods. No obvious irregularities were recorded during either the induction or maintenance of anaesthesia, a finding that compares favourably with the results obtained with cyclopropane (Seevers, Meek, and Rovenstine, 1934 ; Robbins and Baxter, 1937) and with chloroform (Hill, 1932a and b).

A decrease in the voltage of the whole QRST complex but no irregularities were observed when apnoea was produced by high concentrations of fluothane (4.0%) and the administration of the same anaesthetic mixture was continued for a further 90 min. by means of a Starling pump (Fig. 12). This effect cannot be due to stretch or chemical pulmonary reflexes produced by the forced administration of the anaesthetic mixture, because it was not observed in other experiments where the respiratory movements had been stopped with (+)-tubocurarine and maintenance concentrations of the anaesthetic were administered by the same method for periods up to 4 hr. The only possible explanation of this result is the difference in the concentration of anaesthetic in the blood in the two types of experiments.

Cardiac Irregularities Produced by Adrenaline.—Like some other anaesthetics, fluothane was found to increase the sensitivity of the heart to adrenaline. When adrenaline was injected intravenously during fluothane anaesthesia, ventricular fibrillation sometimes occurred. A comparison was made in 13 dogs of the adrenaline hypersensitivity produced by fluothane, chloroform, and cyclopropane. Each dog was anaesthetized to the level of surgical anaesthesia, and increasing doses of adrenaline were injected intravenously at 20 min. intervals. Great care was taken to inject the adrenaline solutions at a constant rate, since in preliminary experiments it was found that the speed of injection was an important factor in the production of heart irregularities. Each dose was therefore dissolved in 5 ml. normal saline and was injected at a rate of 1.0 ml./10 sec. The injections of adrenaline were continued until a dose was found that produced ventricular tachycardia. Sometimes fatal ventricular fibrillation occurred. The dogs that survived the first experiment were allowed to recover and kept under observation for a week. This procedure was then repeated with another anaesthetic, until each dog had been anaesthetized with the three agents. In a few experiments, the dogs were premedicated with morphine or with chlorpromazine. ECG records, lead II, were taken during these experiments.

FIG. 12.—ECG's of a dog recorded during the forced administration of high concentration of fluothane. Records obtained in the experiment of Fig. 6. 1, ECG at the end of 90 min. of inhalation of the maintenance concentration of fluothane 1.2%. 2, ECG at respiratory arrest after 9 min. of inhalation of 4.0% fluothane. 3, ECG taken after 90 min. of respiratory arrest. Fluothane (4.0%) was administered during this time by means of an artificial respiration pump.

This method is essentially that of Meek, Hathaway, and Orth (1937), who assessed their results chiefly on the duration of the ventricular tachycardia produced by a dose of 10 μg./kg. adrenaline. We found it was difficult to use this criterion because our dogs often had more than one period of ventricular tachycardia following the adrenaline injection, and there was a great variation in the doses of adrenaline that produced this

TABLE IV

MEAN DOSES OF ADRENALINE THAT PRODUCE VENTRICULAR TACHYCARDIA IN ANAESTHETIZED DOGS

Numerals in parentheses indicate 95% confidence limits

Anaesthetic	No. of Dogs	Adrenaline (μg./kg.)	Deaths	Remarks
Controls—no anaesthesia	8	17·5 (8–38)		Large variation in the controls; range from 4 to 32 μg./kg.
Fluothane ..	13	8·8 (7–11)	1	
Chloroform ..	9	13·3 (10–17)	1	Unpremedicated
Cyclopropane ..	12	4·3 (3·3–5·4)	2	
Fluothane ..	6	10·1 (7–14)	0	Morphine 2 mg./kg. s.c.
Fluothane ..	4	15·5 (10–23)	0	Chlorpromazine 0·5 mg./kg. i.v.

effect. It was thought better to assess the hypersensitivity by estimating the dose of adrenaline that produced ventricular tachycardia in each dog. A mean dose of 8.8 μg./kg. (−)-adrenaline base injected intravenously produced ventricular tachycardia in unpremedicated dogs anaesthetized with fluothane. The same effect was obtained with 4.3 μg./kg. adrenaline, under cyclopropane, and with 13.3 μg./kg. under chloroform anaesthesia (Table IV). The sensitivity of the heart to adrenaline is therefore increased to a lesser degree by fluothane than it is by cyclopropane, but to a greater degree than by chloroform. If adrenaline was given intramuscularly or subcutaneously, it could be administered in very large doses without producing disturbances of the heart rate or of the conduction mechanism. In some dogs, as much as 200 ml. of 1:100,000 adrenaline was injected subcutaneously in different parts of the body without marked effects. In others, up to 5 ml. of 1:1,000 adrenaline solution was injected intramuscularly into the four limbs, and produced only a slight rise in blood pressure, which was not associated with any cardiac irregularities. Adrenaline is probably absorbed slowly by these routes, and the blood concentration of the amine is never sufficiently high to produce ventricular tachycardia.

The effect of premedication with morphine, and chlorpromazine, was also investigated (Table IV). Morphine did not modify the hypersensitivity to intravenous adrenaline produced by fluothane. On the other hand, chlorpromazine, administered intravenously shortly before the induction, decreased adrenaline hypersensitivity, but it did not protect the dogs completely from the effects of adrenaline.

Equipressor doses of noradrenaline and adrenaline were equally active in producing ventricular tachycardia. This means that the production of ventricular tachycardia and fibrillation in hypersensitive hearts is closely related to hypertension. On the other hand, the intravenous administration of other sympathomimetic amines, such as mephentermine (Wyamine) or methoxamine (Vasoxine) produced rises in the blood pressure comparable to those obtained with adrenaline or with noradrenaline, but without ventricular fibrillation or tachycardia.

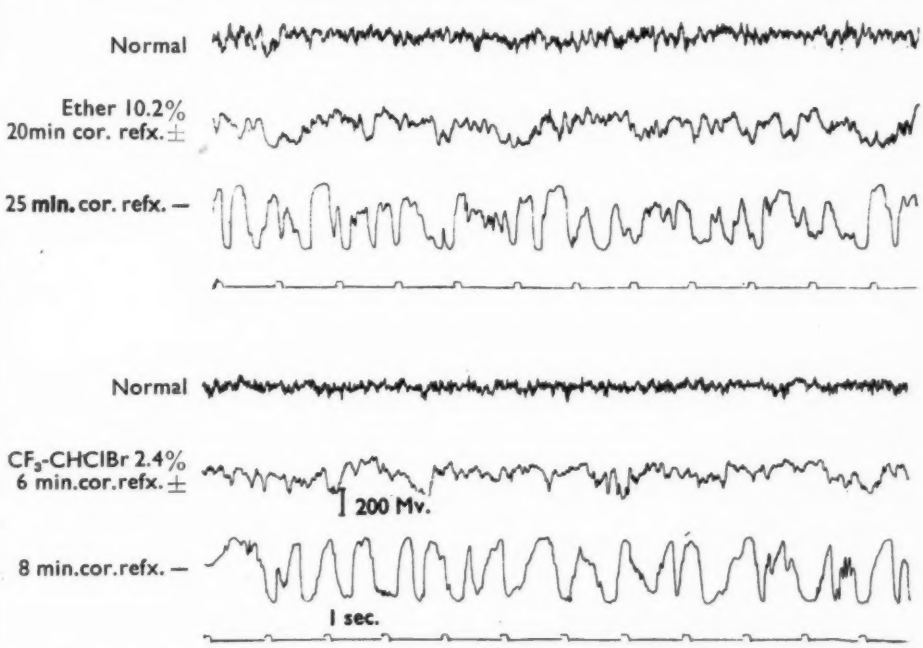


FIG. 13.—Electroencephalograms of a rabbit obtained before and during anaesthesia with ether (top) and with fluothane (bottom).

Electro - encephalography.—Records of the EEG of rabbits with electrodes implanted in the skulls were taken during consciousness, and at different levels of anaesthesia with fluothane. The EEG changes recorded in these experiments were practically the same as those observed in the same animals when anaes-

thetized with ether some days later (Fig. 13).

Salivation, Mucous Secretion, Vomiting and Irritation.—The mouth and trachea of animals anaesthetized with fluothane were always remarkably dry and free from secretion. This is probably due to the ganglion-blocking action of the anaesthetic.

None of the dogs and monkeys anaesthetized with fluothane vomited either during or after its administration. This result contrasts with the high incidence of vomiting, about 50%, found in our laboratories in dogs and monkeys after cyclopropane anaesthesia.

Fluothane applied to the skin did not produce the burning sensation felt after the application of ether or chloroform. Irritation of the mucosa of the mouth and trachea has never been observed during or after the inhalation of its vapours. Its action on the eye has been examined by applying some drops of the liquid in the conjunctival sac and leaving it to evaporate. This produced a fairly intense conjunctivitis and corneal lesions that could be revealed with fluorescine. These lesions, however, were not permanent and no corneal ulcers could be seen two or three days later.

Concentration of Fluothane in the Blood During Anaesthesia.—The concentration of fluothane was estimated in samples of the arterial blood of two dogs and three rabbits taken at different levels of anaesthesia. During induction with 2.4% fluothane in O_2 the concentration of the anaesthetic rose progressively, and in 16 min. had reached an equilibrium at 17.0–22.0 mg./100 ml. (Fig. 14). This blood concentration produced a level of anaesthesia equivalent to Stage II, plane IV, of Guedel's classification. If, at this point, the animals were put on to the maintenance concentration, 0.8%, the concentration of anaesthetic in the blood decreased to about 14.0 mg./100 ml. and remained at this level for as long as the maintenance mixture of fluothane was inhaled. The level of anaesthesia produced by this concentration was equivalent to Stage III, plane II. In some experiments, the concentration of fluothane in the inhaled mixture was increased so as to produce respiratory arrest. The samples of blood taken at this time contained 28–35 mg./100 ml. of the anaesthetic. During recovery from anaesthesia the concentration of fluothane in the blood decreased very rapidly. When the corneal reflex had returned to normal, about 10 min. after the inhalation of the anaesthetic had ceased, its concentration was 7–9 mg./100 ml. Spontaneous movements were observed as a rule 5 min. later, when the concentration had fallen to 4–6 mg./100 ml. No estimations were carried out on samples of blood taken after full recovery.

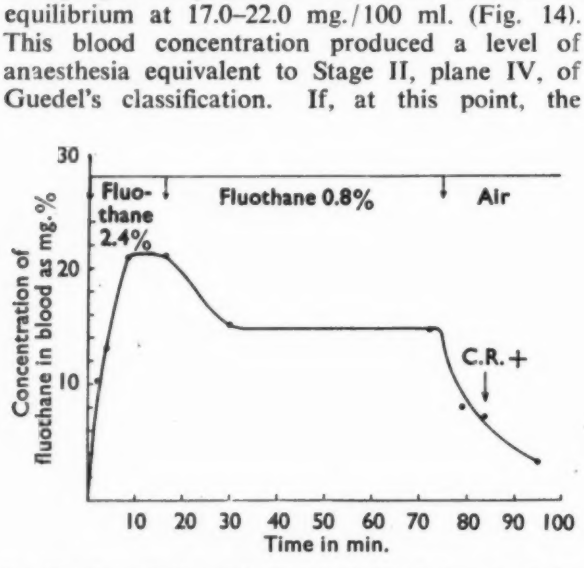


FIG. 14.—Concentration of fluothane in the blood of one dog during anaesthesia. Ordinate, fluothane as mg./100 ml. Abscissa, time in min.

Action of Fluothane on the Isolated Atrium of the Tortoise.—The action of different concentrations of fluothane on the tortoise atrium was compared with the action of ether, chloroform and cyclopropane. An inhibition of the contraction of atrial strips was produced when Ringer solution saturated with these substances was added to the bath.

The average concentrations of the anaesthetics that produced 50% inhibition of the contraction of the tortoise atrium in 8 experiments were: ether, 217 mg./100 ml.; chloroform, 23.7 mg./100 ml.; cyclopropane, 14.3 mg./100 ml.; and fluothane, 38.3 mg./100 ml. It is evident that both ether and fluothane inhibit the heart only when their concentration in the bath is higher than that found in dogs during anaesthesia so deep as to produce respiratory arrest, i.e., 30 mg.% and 150 mg.% respectively.

Action of Fluothane on the Liver.—Although in the experiments described above no delayed deaths attributable to the action of fluothane were observed, two liver function tests were undertaken. Two batches of 4 rats each were anaesthetized for 1 hr. with fluothane every day for 5 consecutive days, and the hippuric acid excretion test was carried out before the first, and immediately after the last, period of anaesthesia. The amount of hippuric acid excreted in the urine was the same before and after repeated anaesthesia.

A similar negative result was found in a dog by the bromosulphalein method. In the tests carried out before and after repeated periods of anaesthesia with fluothane, no significant differences

were observed either in the retention of the dye in the blood or in the rate of its disappearance. These results suggest that hepatic function is not affected by anaesthesia with fluothane.

Renal Function Tests.—The renal function of rats was examined by the water diuresis and phenol red excretion tests. Seven batches of 4 rats each were anaesthetized 5 times with fluothane for 1 hr. on consecutive days and both tests were carried out, in different batches of animals, before and immediately after the periods of anaesthesia. No significant difference was found between the results of the control tests and those carried out after anaesthesia. The renal function of one dog was examined by the phenol red excretion method once before the animal was anaesthetized (twice for 3 hr. on two consecutive days) and again 24 hr. after the second period of anaesthesia. The excretion of phenol red was unaltered.

Histopathology. — Histopathological observations were made of the tissues of 20 untreated rats, 6 rats anaesthetized with ether, and 30 rats anaesthetized with fluothane for 45 min. on each of 5 consecutive days; 2 dogs anaesthetized with fluothane for 6 hr., and one dog anaesthetized for 1½ hr. with a concentration that produced respiratory arrest; and 1 untreated monkey, 1 monkey anaesthetized for 2 hr. with chloroform, and 4 monkeys anaesthetized with fluothane once for periods up to 6 hr. and one monkey anaesthetized 6 times for 3 hr. on alternate days.

Dr. G. E. Paget, who examined the sections of the specimens from these animals, reported: "The most consistent histopathological change seen in animals treated with fluothane occurs in the kidney. It consists of a dilatation of the proximal convoluted tubules and is associated with slight cytological changes in the cells of these tubules (Fig. 15).

"The changes are more prominent in animals subjected to repeated prolonged anaesthesia with this agent, but it can be seen in animals killed after only one period of anaesthesia. No necrosis of renal tissue was seen in any of the animals. These changes must be taken to indicate mild damage to the proximal convoluted tubule cells. In order to assess the significance of these changes, histochemical studies were made on kidneys from animals treated with fluothane. These showed that no significant change occurs in the quantity and distribution of alkaline phosphatase or of non-specific esterase in the dilated tubules. In view of this and of the absence of necrosis, it seems probable that the changes do not indicate any serious or irreversible damage to the kidney. However,

FIG. 15A

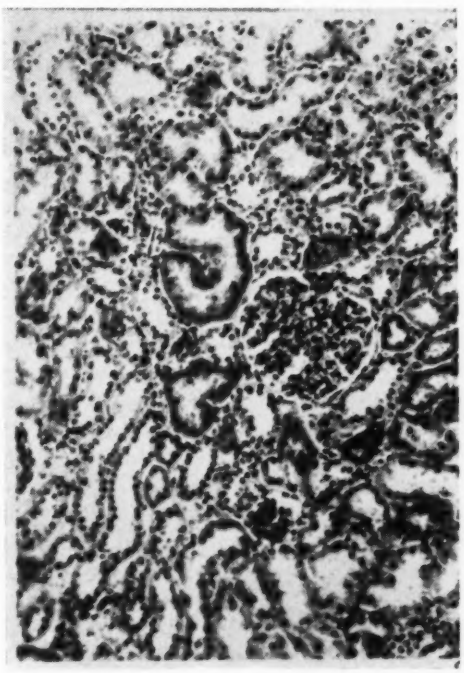


FIG. 15B

FIG. 15.—A = Section of the kidney of a monkey two days following one period of anaesthesia with fluothane. B = Section of kidney of a normal monkey. H. & E., × 120.

until something is known of the action of this agent in man, it would be wise to avoid its use in the presence of severely impaired renal function.

"There are also minor changes in the liver. These are of trivial extent and degree compared with those seen in a monkey anaesthetized on one occasion with chloroform or with the changes known to occur in man after chloroform anaesthesia.

"The lungs of all rats show varying degrees of naturally occurring chronic interstitial pneumonia. No consistent changes attributable to the administration of the anaesthetic can be detected in the lungs of rats or other animals.

"No changes due to administration of this anaesthetic agent were seen in any other organ examined. It thus seems that, apart from mild renal changes, this agent produces little toxic damage to tissues, and, apart from the previously-mentioned proviso concerning impaired renal function, there is, from this evidence, no contra-indication to its use in man."

From this report it is clear that the only obvious pathological change found in the tissues of animals treated with fluothane is a dilatation of the proximal convoluted renal tubules and slight cytological changes in their cells. It is probable that this mild lesion is of no great importance because (1) renal necrosis has never been observed in these animals, (2) the change is not associated with any alteration in the amounts and distribution of two enzymes of the tubules, and (3) the renal functions, as measured by the phenol-red excretion and water-diuresis tests, were not altered even immediately after repeated anaesthesia.

On the other hand, Eschenbrenner (1944) and Eschenbrenner and Miller (1945) found extensive tubular necrosis in the kidneys of adult male mice anaesthetized for long periods with chloroform. Hewitt (1956) has been able to produce this lesion in some strains (CBA) of adult male mice kept for 2 hours in concentrations of chloroform (4 mg./l. =0.078%) too low to produce any signs of anaesthesia. No lesions were found in the kidneys of female mice of these sensitive strains after the same treatment with chloroform. The production of renal necrosis with sub-anaesthetic concentrations of chloroform has been confirmed in our laboratories, but we have been unable to observe similar results in the kidneys of male mice after they have been exposed for 2 and 4 hours to similar concentrations of fluothane (0.075%).

Microscopical examination of the livers of animals anaesthetized for long periods, or repeatedly, with fluothane showed only minor changes, a result that has been corroborated by biochemical tests. In no experiment was fatty degeneration of the liver found, a very frequent result of chloroform and divinyl ether anaesthesia. The fatty degeneration produced by chloroform anaesthesia, which is a classic example of delayed toxic action, can be obtained with very low concentrations of the agent. Hewitt (1956), and ourselves, have found it in mice, males and females, that had been exposed to chloroform in concentrations of about 0.078% for 2 hr. No hepatic lesions have been found in mice after being exposed to similar concentrations of fluothane.

Summary

1. Fluothane ($CF_3CHClBr$) is a volatile liquid with a b.p. of 50.2° C. and an S.G. of 1.86. It is not inflammable when mixed with O_2 in concentrations from 0.5 to 50% (v/v). It is stable over soda lime.

2. It is an inhalation anaesthetic more potent than ether and choloroform on experimental animals. Its therapeutic ratio is about twice that of ether. Induction of anaesthesia and recovery are both rapid and free from excitement. It produces good muscular relaxation. It does not cause salivation or vomiting.

3. With the exception of hypotension, it does not produce any serious functional disturbances. It does not produce cardiac irregularities, but increases the sensitivity of the heart to adrenaline. It does not increase capillary bleeding.

4. The inhalation of high concentrations (3.5%) stops the respiration, but this apnoea is easily reversible.

5. The only pathological lesion found in animals after its use is a mild dilatation of the proximal tubules of the kidney. This lesion is not associated with alteration of the renal function.

Clinical trials on fluothane are being carried out by Dr. M. Johnstone. An account of his results on 500 patients has been published (Johnstone, 1956) since this paper was submitted for publication.

The author is grateful to Dr. C. W. Suckling and other members of the staff of the Research Laboratories of the General Chemicals Division of I.C.I. at Widnes for preparing the fluothane used in these experiments. He would like also to thank his colleagues, Dr. R. R. Goodall, for developing the method for the estimation of fluothane in blood, Dr. J. M. Pryce, for the estimation of some of its physical constants, Mr. J. M. Thorp, for the measurements of the cardiac outputs, and Dr. G. E. Paget, for the pathological examination of animals anaesthetized with fluothane. Finally, he would like to acknowledge the technical help given by Mr. J. Dee.

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APPENDIX

ESTIMATION OF FLUOTHANE IN BLOOD

By R. R. GOODALL

For the estimation of fluothane in blood, extracts in petroleum ether were heated in sealed ampoules with sodium amoxide. Bromide was released and determined nephelometrically as silver bromide. The hydrolytic reaction was limited to 1½ hr., since prolonged heating caused solution of chloride from the glass. It was essential to hydrolyse samples and standards together in the same pressure vessels, as the reaction was incomplete (about 60% of theory).

Special Reagents

Sodium in Amyl Alcohol.—Place 50 ml. of amyl alcohol (A.R. quality) in a stainless steel beaker and add 1.1 ±0.1 g. of clean sodium (free from halide) in small pieces. Cover the beaker with a petri dish during solution and warm if the last small pieces of sodium are slow to dissolve.

Standard Solution of $CF_3CHBrCl$.—Place 100 ml. of distilled water in a 4 oz. liquid bottle with a well-fitting glass stopper. Chill the bottle and contents in an ice-bath. Weigh accurately anaesthetic (90–110 mg.) in a tared capillary pipette having a rubber teat at the top. Deliver the contents of the pipette under the surface of the water in the bottle and rinse the pipette several times by pressing and releasing the rubber teat. Leave the contents of the bottle unshaken in the ice-bath until just before use. Then shake for one minute to dissolve the droplets of $CF_3CHBrCl$. Anaesthetic vapour is lost very readily from aqueous solution, and it is essential to use a bottle that is almost full of liquid. It is now known that fluothane is less readily lost from petroleum ether, and standards might advantageously be prepared in this solvent.

Extraction

Standards.—Into 10 ml. tubes (B14 stoppered) place 4 ml. of petroleum ether (b.p. 100–120° C.). In successive tubes place 5, 4.5, 4.0 and 3.5 ml. of water and then 0, 0.5, 1.0 and 1.50 ml. of the standard aqueous anaesthetic solution, delivered from 0.50 and 1.00 ml. Folin–Ostwald pipettes filled by immersion and *not* by suction. Stopper each tube and shake for 2 min. Allow the layers to separate. Then from each tube withdraw a 3.5 ml. aliquot of the petrol layer and place in a 10 ml. ampoule into which 2.0 ml. of the sodium amoxide solution has been introduced. Seal each ampoule.

Prepare in duplicate three tubes each containing petroleum ether (4 ml.) and normal blood (4 ml.). In successive tubes, place water (1, 0.5, and 0 ml.) followed by standard anaesthetic solution (0, 0.5, and 1.0 ml. from Folin–Ostwald pipettes). Extract by shaking for 5 min., centrifuge at 2,000 rev./min. for 2 min. with stoppers removed; then remove a 3.5 ml. aliquot of each petrol layer and continue as described above.

Tests.—For each test, place 4 ml. of petroleum ether and 1 ml. of water in an appropriate 10 ml. tube. Place 4 ml. of the blood sample (or less if the concentration of $CF_3CHBrCl$ is expected to exceed 25 mg. % w/v) under the surface of the petroleum. If required, add more blood from an untreated animal to the tube until the total blood volume is 4.0 ml. Extract, centrifuge, and transfer 3.5 ml. aliquots to ampoules containing sodium amoxide and seal as described above.

Hydrolysis

The ampoules are heated in a pressure cooker at 15 lb./sq. in. for 1½ hr.

Determination of Halide as Silver Halide.—Open each ampoule and transfer the contents by shaking into a 6 in. by ⅝ in. test-tube. Rinse each ampoule with N-sulphuric acid (volume, 0.5 ml. more than that required to neutralize the sodium amoxide) followed by sufficient water to make the total aqueous layer 8 ml., and mix well. After settling, remove the lower (aqueous layer) to a clean tube, add 2 ml. 0.01N-silver nitrate, stir momentarily, and transfer immediately to a dark cupboard for 10 min. Then read quickly the optical density of each colloidal suspension of silver halide in a 1 cm. cell at 520 mμ against water, using a Unicam S.P. Spectrophotometer. (Note: After adding the silver nitrate

avoid unnecessary exposure to bright light.) Plot the optical density of the aqueous and blood standards against the known concentration. From the observed optical density of each test obtain the concentration on the aqueous standard graph and multiply the result by the ratio of aqueous/blood slopes.

Optical densities of extracts of aqueous and blood standards are exemplified in the following table:

Mg.% w v in Original Solution		d_1 cm.	at 520 mμ
Aqueous	0	0·10	0·11
,,	13·3	0·30	0·31
,,	26·5	0·47	0·51
,,	39·8	0·76	0·77
Blood	0	0·08	0·09
,,	13·3	0·25	0·26
,,	26·5	0·43	0·46

Note added in proof: An improved method, using reduction by lithium aluminium hydride to release halide from fluothane, is being developed.

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THE EFFECTS OF RESERPINE AND COMPOUND 48/80 ON THE RELEASE OF AMINES FROM THE MAST CELLS OF RATS

BY

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(RECEIVED JUNE 28, 1956)

Recently we have found that histamine liberators like compound 48/80, morphine and propamidine release not only histamine but 5-hydroxytryptamine (5HT) as well, from the perfused hindquarters of rats (Bhattacharya and Lewis, 1956). The mast cells of the skin and subcutaneous tissue were thought to be the source of both the histamine and the 5HT since, in the rat at least, they contain both amines (Riley and West, 1953; Benditt, Wong, Arose, and Roeper, 1955; Parratt and West, 1956). Further, Pletscher, Shore, and Brodie (1956) have shown that reserpine has the property of depleting a number of organs such as intestinal tract, brain and platelets of their 5HT, which in these organs is not associated with mast cells. In the present experiments, therefore, we have examined the effect of reserpine on the 5HT content of mast cells in skin and subcutaneous tissue.

Reserpine was either injected into perfused hindquarters of rats to ascertain if, like 48/80, it released 5HT or histamine or both; or rats were treated with intraperitoneal injections of reserpine and then 48/80 was injected into their perfused hindquarters and the amounts of histamine and 5HT released were compared with those released from the hindquarters of untreated rats and of rats previously treated with intraperitoneal injections of 48/80 or morphine. In addition, the effect of reserpine on mast cells in the mesentery and subcutaneous tissue was studied histologically and compared with that of 48/80.

METHODS

Rats weighing 130 to 160 g. were anaesthetized with intraperitoneal injections of 40 mg./kg. pentobarbitone sodium. The hindquarters were perfused with oxygenated Locke solution through the abdominal aorta and the venous effluent was collected from the vena cava. The venous effluent was tested for histamine on the atropinized guinea-pig ileum and for 5HT on the atropinized rat's colon, as described by Feldberg and Toh (1953). All values of 5HT and histamine refer to base. Details of the perfusion and assay have been given elsewhere (Bhattacharya and Lewis, 1956). All substances injected arterially were given in a volume of 0.5 ml. The standard dose of 48/80 used in studying the release of 5HT and histamine was 100 μg. Reserpine (Light & Co.) was dissolved, after adding one drop of glacial acetic acid, in a mixture of propylene glycol, alcohol, and water in the ratio of 1:1:6 to give a concentration of 5 mg./ml., and was diluted with distilled water when injected in lower concentrations. Morphine was used as sulphate; doses injected refer to the salt.

Studies of Mast Cells of Subcutaneous Tissue and Mesentery.—Spreads were made of both tissues within a few minutes after death. The subcutaneous tissue was taken from the loose areolar tissue of the interscapular region. The spreads were fixed and stained with toluidine blue according to the procedure described by Riley (1953).

RESULTS

The previous finding, that 48/80 releases 5HT as well as histamine from the perfused hindquarters of the rat, has been confirmed. In Table I the results of four experiments are given in which 100 μg. 48/80 was injected intra-arterially into the perfused hindquarters. The amounts of histamine released varied between 85.3 and 102 μg. (mean 91.3 μg.), those of 5HT between 4.3 and 8 μg. (mean 5.8 μg.).

Intra-arterial injections of 0.5 to 2 mg. of reserpine released neither histamine nor 5HT from the perfused tissues.

Release of Histamine and of 5HT by 100 μg. 48/80 from the Perfused Hindquarters of Rats Previously Treated with Intraperitoneal Injections of 48/80, Morphine, or Reserpine

Previous Treatment with 48/80.—After repeated intraperitoneal injections of 48/80 the amounts of histamine, as well as of 5HT, released from the perfused hindquarters by an intra-arterial injec-

TABLE I

RELEASE OF HISTAMINE AND 5-HYDROXYTRYPTAMINE BY INTRA-ARTERIAL INJECTION OF 100 μG. 48/80 FROM PERFUSED HINDQUARTERS OF RAT AFTER REPEATED INTRAPERITONEAL INJECTIONS OF 48/80

No. of Days during which 48/80 was Injected	Output in μg. of	
	5HT	Histamine
0	8·0	86
0	5·5	85
0	4·3	92
0	5·2	100
1	1·1	8·5
1	1·3	37
1	1·8	32
2	0·4	2·7
3	0·7	5·3
4	3·0	7·4
7	0·8	4·7
8	0·8	8·7
9	1·0	14
10	0·5	9·7

tion of 100 μg. 48/80 were much smaller than those released from the perfused hindquarters of normal rats. This is shown in Table I. The rats received daily two intraperitoneal injections of 48/80, one in the morning and one in the evening. The first dose was 100 μg., and was increased with each injection by 50 μg. up to 500 μg., so that after the fourth day the rats were given 500 μg. twice daily. The perfusion was carried out in the morning following the last injection. The results of Table I show that the intraperitoneal treatment with 48/80 for 1 day is sufficient to reduce greatly the amounts of histamine and 5HT released by the intra-arterial injection of 100 μg. 48/80 into the perfused hindquarters. The mean amounts released in the three experiments given in Table I were about a quarter to a third of those released from untreated rats. There was further reduction in the amounts of histamine and 5HT released from rats which had been treated for two days with intraperitoneal injections of 48/80. In the experiment given in Table I the amount of histamine released was about 3% and that of 5HT about 7% of the mean amounts released from untreated rats. Further treatment with intraperitoneal injections of 48/80 for longer periods and with higher doses, however, did not cause a further reduction.

Feldberg and Talesnik (1953) have shown that the histamine content of skeletal muscle and skin of rats, when reduced by repeated intraperitoneal injections of 48/80, remains low for several days and then rises gradually in the following weeks. In the present experiments the effect of intra-arterial injections of 48/80 was studied on the histamine and 5HT output from the perfused hindquarters of rats which were first treated for 3 or 10 days with repeated intraperitoneal injections of 48/80 and were then allowed to recover. The results are shown in Table II. The first column gives the days of treatment, the second the number of days between the last intraperitoneal injection and the perfusion, and the third and fourth columns the amounts of 5HT and histamine released by the intra-arterial injection of 100 μg. 48/80 into the perfused hindquarters. The amounts are given in μg. and are also expressed as % of the average amounts released from the perfused hindquarters of untreated rats, i.e., the first four experiments of Table I.

There is a definite increase in the amounts of histamine and 5HT released from the perfused hindquarters when 11 days or more have elapsed between the last intraperitoneal injection and the perfusion. However, even when the perfusion is carried out 24 or 33 days after the last intraperitoneal injection, the amounts of histamine and 5HT released are only half or less than the amounts released from the hindquarters of untreated rats.

Previous Treatment with Morphine.—The results obtained from four rats which were given daily two intraperitoneal injections of morphine are

TABLE II

RELEASE OF HISTAMINE AND 5HT BY INTRA-ARTERIAL INJECTION OF 100 μG. 48/80 FROM PERFUSED HINDQUARTERS OF RATS PREVIOUSLY TREATED WITH INTRAPERITONEAL INJECTIONS OF 48/80

Days of Treatment with 48/80	Days between last 48/80 Injection and Perfusion	Output of 5HT		Output of Histamine	
		(In μg.)	(As % of Normal)	(In μg.)	(As % of Normal)
0	—	5·8	100	91	100
3	1	0·7	12	5·3	5·7
3	11	1·1	19	26	29
3	17	1·6	28	57	61
10	1	0·5	9	9·7	11
10	12	1·2	21	25	28
10	18	2·3	40	27	29
10	24	3·3	57	41	44
10	33	2·2	38	37	39

TABLE III

RELEASE OF HISTAMINE AND 5HT BY INTRA-ARTERIAL INJECTION OF 100 μG. 48/80 FROM PERFUSED HINDQUARTERS OF RATS PREVIOUSLY INJECTED INTRAPERITONEALLY WITH MORPHINE

Pretreatment with Morphine		Output in μg. of	
No. of Days Treated	Total Morphine Injected (mg./kg.)	5HT	Histamine
1	20	4·5	110
2	50	2·4	101
3	80	2·2	48
3	80	2·8	67

shown in Table III. On the first day 10 mg./kg. was injected each time, subsequently the dose was increased to 15 mg./kg.

The release of 5HT and histamine by the arterial injection of 100 μg. 48/80 into the perfused hindquarters of one of these rats, which had been treated with morphine for one day only, was of the same order as that observed in control rats. In the rats treated with morphine for 3 days there was a definite reduction in the amounts of 5HT and histamine released from the perfused hindquarters by 48/80.

Previous Treatment with Reserpine.—Rats were injected intraperitoneally with 5 mg./kg. reserpine and intra-arterial injections of 100 μg. 48/80 were made into their perfused hindquarters 3, 6 or 18 hr. later. The amounts of histamine released were normal, and in the experiments at 3 and 6 hr. 48/80 also released the usual amounts of 5HT. However, in the experiment in which perfusion was carried out 18 hr. after the reserpine injection, the release of 5HT by 48/80 was reduced (Table IV).

TABLE IV

RELEASE OF HISTAMINE AND 5HT BY INTRA-ARTERIAL INJECTION OF 100 μG. 48/80 FROM PERFUSED HINDQUARTERS OF RATS PREVIOUSLY INJECTED INTRAPERITONEALLY WITH RESERPINE

Pretreatment with Reserpine		Interval in Hr. between Reserpine Injection and Injection of 48/80	Output in μg. of	
mg./kg./ Injection	No. of Injections		5HT	Histamine
5	1	3	5·9	107
5	1	6	4·4	110
5	1	18	2·0	117
3	2*	18	0·6	106
3	3*	18	0·5	86
3	3*	18	1·0	89

* One injection daily.

In a second series of experiments three rats were given intraperitoneal injections of 3 mg./kg. reserpine on two or three successive days and the hindquarters perfused 18 hr. after the last injection. As shown in Table IV the 48/80 again released the usual amounts of histamine but only about 10 to 20% of the amounts of 5HT released from the hindquarters of untreated rats. Compound 48/80 released the usual amounts of both histamine and 5HT from rats treated with 1 ml./kg. of the reserpine solvent.

Effect of Intraperitoneal Injections of 48/80, Morphine, and Reserpine on Mast Cells in Subcutaneous Tissue and in the Mesentery

Compound 48/80.—The intraperitoneal injections of 48/80 affected the mast cells of the mesentery more readily than those of the subcutaneous tissue. After two intraperitoneal injections of 100 and 150 μg. 48/80 on the same day less than half of the mast cells of the subcutaneous tissue were disrupted (Fig. 1b), whereas in the mesentery practically all were totally disrupted. This difference might well be due to the high concentration of 48/80 in the peritoneal cavity. After two intraperitoneal injections of 48/80 each day for three days, doses being increased from 100 μg. by 50 μg. with each injection, the subcutaneous tissue also contained only about 5–10% of the normal number of mast cells and they were partly disrupted (Fig. 1c). In rats in which intraperitoneal injections of 48/80 were continued for 10 days by increasing the dose given per injection up to 500 μg., no further damage occurred.

It required several days after cessation of the injections of 48/80 before the reappearance of the mast cells was observed. Three rats had been treated with intraperitoneal injections of 48/80 for three days and were allowed to survive for 3, 5 and 11 days. The recovery of the mast cells in the subcutaneous tissue and mesentery was the same; after three days there was no recovery, after 5 days some, and after 11 days there was nearly complete recovery (Fig. 1); the number of mast cells was nearly normal, but some were not heavily granulated. In rats which had been treated for 10 days with intraperitoneal injections of 48/80 recovery of the mast cells in the mesentery was more delayed than that in the subcutaneous tissue. In the latter the mast cells had fully recovered 12 days after the last injection; in fact the number present seemed to be greater than usually found in untreated rats; on the other hand there were no mast cells, or only a few, in the mesentery even 18 days after the last injection.

Morphine.—The mast cells of the mesentery were more affected by intraperitoneal injections of morphine than the mast cells of the subcutaneous tissue. After 1 to 3 days' treatment with morphine injections twice daily in a dose of 10 mg./kg./injection on the first day and 15 mg./kg. subsequently, the mast cells of the subcutaneous tissue showed a normal appearance and their number had not decreased. In the mesentery, however, many disrupted mast cells were encountered even after only 1 day's treatment with morphine, and after 2 and 3 days' treatment the number of mast cells was greatly reduced and those present were disrupted.

Reserpine.—The mast cells of the rats treated with intraperitoneal injections of reserpine and used for the experiments concerned with the release of 5HT and histamine showed no disrup-

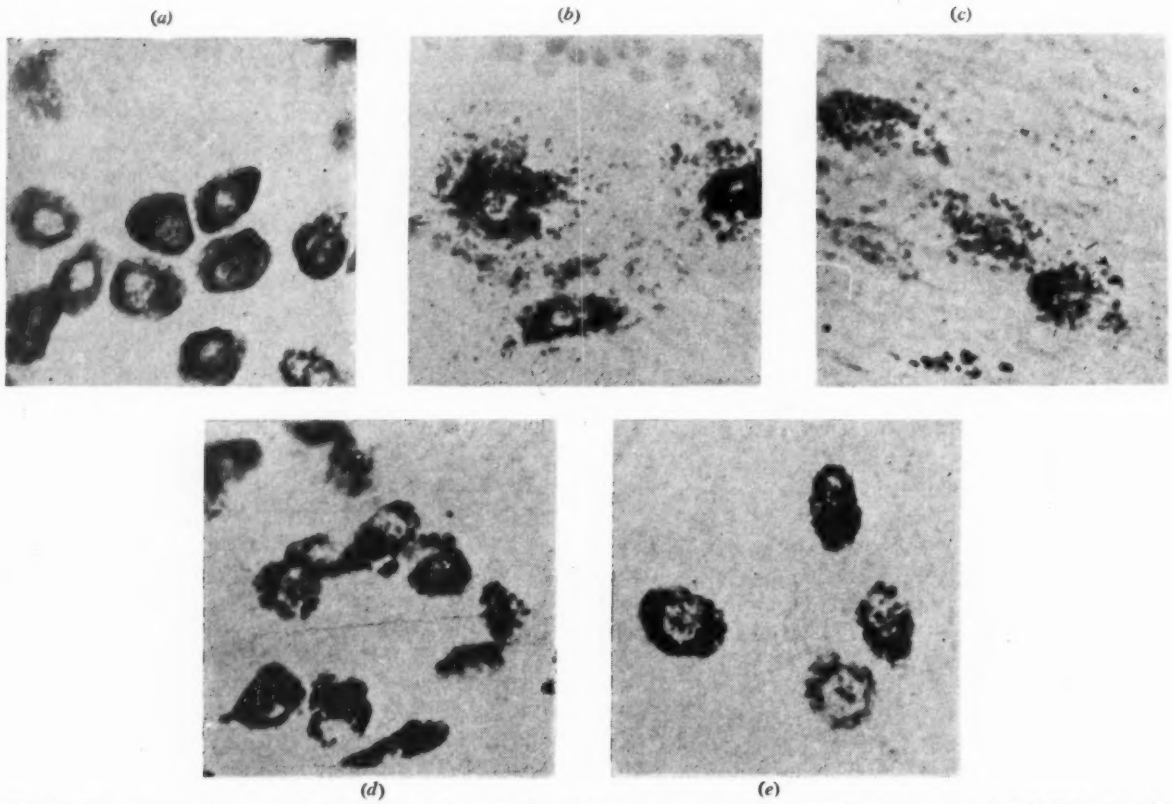


FIG. 1.—Subcutaneous tissue spreads from untreated rats in (*a*), and from rats treated with intraperitoneal injections of 48/80 for 1 day in (*b*), and for 3 days in (*c*). In (*d*) and (*e*), 5 and 11 days respectively had elapsed since the treatment with intraperitoneal injections of 48/80 for 3 days. The spreads were stained with toluidine blue. Magnification, × 680.

tion either in the subcutaneous tissue (Fig. 2) or in the mesentery, and the number of mast cells present in both these tissues was normal.

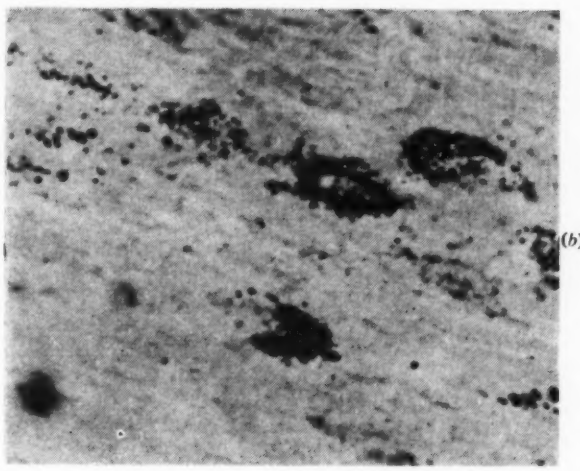
However, as seen in Fig. 2, there is a slight change in the appearance of the mast cells in rats treated with intraperitoneal injections of reserpine for 3 days as compared with those in normal rats. The reserpine treatment causes some swelling and degranulation of the mast cells, changes observed by Riley and West (1955) when they examined the effect on mast cells of small doses of 48/80.

DISCUSSION

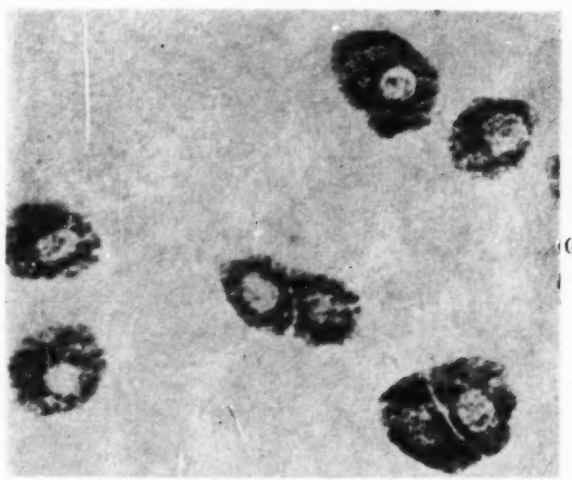
The results of the present experiments show that both 48/80 and reserpine act on the mast cells of rats but that they act in a different way. 48/80 disrupts the mast cells and in doing so releases the histamine and 5HT they contain. This action of 48/80 is an immediate one and has been termed explosive. Reserpine does not disrupt the mast cells, nor does it release their histamine, but it depletes them of their 5HT as it depletes other tissues like brain, intestine, and platelets of this amine. Further, the action of reserpine on the mast cells differs from that of 48/80 in that it requires several hours before a depletion of 5HT occurs.

These conclusions were derived not from experiments in which the 5HT and histamine content of the tissues was assayed, but by determining the amounts of histamine and 5HT released by 48/80 from the perfused hindquarters of normal rats and of rats previously treated with intraperitoneal injections of either 48/80 or reserpine. Injections of 48/80 into the artery of the perfused hindquarters of normal rats led to the appearance in the venous effluent of relatively large amounts of histamine and 5HT, whereas reserpine similarly injected had no effect of this kind. In these acute experiments there was therefore no indication of an effect of reserpine on the amine content of the mast cells On the other hand it was possible with intraperitoneal injections of reserpine to reduce the amount of 5HT released from the mast cells by 48/80 provided sufficient time was allowed for the reserpine to act. This effect of reserpine, which was brought about without disruption of

(*a*)



(*b*)



(*c*)

FIG. 2.—Subcutaneous tissue spreads from untreated rats in (*a*), and from rats treated for three days with intraperiotneal injections of 48/80 (doses as in text) (*b*), and reserpine 3 mg./kg. daily in (*c*). The spreads were stained with toluidine blue. Magnification, × 680.

the mast cells and without concomitant release of histamine, was shown when 48/80 was injected into the perfused hindquarters of rats treated with intraperitoneal injections of reserpine. The 48/80 released normal amounts of histamine but traces only of 5HT, and the mast cells were shown not to be disrupted. Intraperitoneal injection of reserpine thus acts entirely differently from that of 48/80 which was shown to disrupt the mast cells and release both their histamine and their 5HT.

The finding that reserpine requires several hours to affect the 5HT content of the mast cells is in agreement with the action of reserpine in depleting other tissues of their 5HT. According to Pletscher *et al.* (1956) there is also a latency of hours before the 5HT content of brain or intestinal tract is reduced by an injection of reserpine.

Experiments on the recovery of mast cells after disruption by 48/80 show that, when they have reappeared, 48/80 does not release the normal amounts of histamine and 5HT from them despite the fact that the histological picture is normal. Twelve days after prolonged treatment with intraperitoneal injections of 48/80, the mast cells were again present in the subcutaneous tissue, normal in number and appearance. Nevertheless at this stage of recovery, only small amounts of histamine and 5HT were released by 48/80 from the perfused hindquarters. This may mean that the mast cells in their early stages of recovery are either more resistant to the action of 48/80 or that they do not yet contain their full complement of amines.

The finding, also reported by Riley and West (1955), that, after prolonged treatment with intraperioneal injections of 48/80, when the mast cells have practically disappeared from the subcutaneous tissue and mesentery, they return in the subcutaneous tissue much earlier than in the mesentery, is difficult to explain. The fact that 48/80 is injected into the peritoneal cavity may mean that it can act on the mesentery in a much higher concentration than on the subcutaneous tissue, which it reaches through the blood stream, and that the high concentration exerted for long periods in the mesentery not only disrupts mast cells but affects their precursors as well.

SUMMARY

1. Reserpine released neither histamine nor 5-hydroxytryptamine (5HT) when injected into the perfused rat hindquarters under conditions in which 48/80 released large amounts of both amines.

2. 48/80 released only small amounts of both histamine and 5HT from the perfused hindquarters

of rats previously treated with intraperitoneal injections of 48/80. In these rats the mast cells of the mesentery and subcutaneous tissue were disrupted and had disappeared.

3. 48/80 released large amounts of histamine but only small amounts of 5HT from the perfused hindquarters of rats previously treated with intraperitoneal injections of reserpine. In these rats the appearance of the mast cells was slightly different from those in normal rats, but they were not disrupted and were present in normal number.

4. Whereas 48/80 acts on mast cells, disrupting them, thereby releasing the histamine and 5HT they contain, reserpine does not disrupt them and specifically depletes them of 5HT, as it depletes other tissues of this amine.

This work was carried out during the tenure, by G. P. L., of a Beit Memorial Fellowship.

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OXIMES AND HYDROXAMIC ACIDS AS ANTIDOTES IN ANTICHOLINESTERASE POISONING

BY

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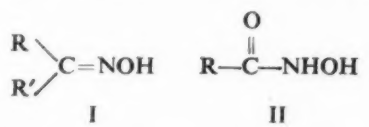
It has recently been shown *in vitro* that oximes (I) and hydroxamic acids (II) will both react with and reactivate cholinesterase (ChE) inhibited by



I II

certain anticholinesterase (antiChE) compounds (Hackley, Plapinger, Stolberg, and Wagner-Jauregg, 1955; Wilson, Ginsburg, and Meislich, 1955; Childs, Davies, Green, and Rutland, 1955). In addition, Holmes and Robins (1955) have shown that certain oximes will reverse neuromuscular block following the action of these inhibitors. Simultaneously with these studies, those oximes and hydroxamic acids which appeared to be the most promising in the above tests were examined in the intact animal for their effectiveness in antiChE poisoning. From preliminary results, however, it became apparent that there was not always a close correlation between *in vitro* tests and *in vivo* behaviour. A comprehensive screening *in vivo* of all available oximes and hydroxamic acids was therefore undertaken. This involved examining the compounds for their toxicity and for their protective effect against *iso*propyl methylphosphonofluoridate (Sarin) poisoning in the rat, each compound being given (a) before and (b) immediately following the inhibitor.

Those oximes which appeared from the screening tests to give the greatest degree of protection against sarin poisoning have been studied in greater detail, both in other species against sarin and also in the rat against tetraethylpyrophosphate (TEPP) and di*iso*propyl phosphorofluoridate (dyflos).

METHODS

In the preliminary examination of oximes and hydroxamic acids all the tests were carried out on female albino rats of 180–200 g. In the more detailed experiments mice (18–22 g.), guinea-pigs (230–380 g.), rabbits (1.3–1.7 kg.) and monkeys (2.7–5.0 kg.) were also employed. Except with monkeys, where both sexes were used, the animals were all female.

The oximes and hydroxamic acids were dissolved in water and given by intraperitoneal injection, the antiChE compounds being diluted with saline and given by subcutaneous injection.

Toxicity Screening in Rats.—Because the object of this investigation was to search for potential therapeutic agents for antiChE poisoning, an arbitrary upper limit of test dose was set at 150 mg./kg. Since at this dose many oximes proved toxic, a range of doses was used down to a lower limit of 13 mg./kg., the ratio between successive doses being 1.5. Two rats were tested at each dose level. By this screening procedure the maximum dose (up to the limit of 150 mg./kg.) which produced no obvious effects was determined. The compounds were given by intraperitoneal injection, the volume of injection varying according to the solubility of the substance. It was never greater than 5 ml., which appeared to be well tolerated.

Screening of Compounds against Sarin in Rats.—Two types of experiment were carried out in which the oximes and hydroxamic acids were tested against twice the LD50 of sarin (0.24 mg./kg.). In the first the compound was given 15 min. before the sarin, and this test has been termed "prophylactic." The second test, in which the compound was given 30 sec. after the inhibitor, has been called "therapeutic." (The designation of these tests as "prophylactic" and "therapeutic" is made for convenience and does not in any way imply the mechanism of action of the compound.) In both tests groups of 5 rats were used. The oximes and hydroxamic acids were given intraperitoneally at the previously determined maximum dose which produced no signs. The sarin was given by subcutaneous injection at a standard volume of 1 ml./kg. The number of dead up to 48 hr. and the time interval to death were used in the estimation of the effectiveness of the compounds tested.

Further Experiments on Oximes.—In order to investigate more fully the effect in antiChE poisoning of those oximes which proved the most promising against sarin in the screening tests, experiments were

carried out to determine the LD50 values of antiChE compounds with the administration of these oximes under different conditions. In all experiments, with the exception of that with monkeys, the inhibitors were given by subcutaneous injection at a standard volume of 10 ml./kg. for mice, 1 ml./kg. for rats and guinea-pigs, and 0.5 ml./kg. for rabbits. With monkeys where two animals were used at each dose level the inhibitor was given by intravenous injection at a volume of 1 ml./5 kg. With all other species four animals were used at each dose level. The ratio between successive doses was 1.26.

LD50 values were normally calculated by the method of moving averages (Thompson, 1947), using the tables constructed by Weil (1952). Where this was not possible the technique of probit analysis (Finney, 1947) was employed. With very few exceptions estimates of LD50 were determined at least twice and, except where indicated in the text, each LD50 value is the mean of duplicate or triplicate estimations.

Materials.—The antiChE compound used mainly throughout this work was sarin. In some of the later experiments TEPP and dyflos were also employed. The oximes and hydroxamic acids were obtained either from commercial sources or were synthesized at this establishment. They were all over 95% pure.

Results

Toxicity of the Oximes and Hydroxamic Acids to Rats.—The toxicity of 23 oximes and 9 hydroxamic acids has been determined. Table I lists the maximum dose levels of oximes at which no effects were observed. In addition to these oximes, the following hydroxamic acids were tested: glycinehydroxamic acid, hippurohydroxamic acid, nicotinhydroxamic acid, nicotinhydroxamic acid methiodide, *m*-nitrobenzhydroxamic acid, salicylhydroxamic acid, tropohydroxamic acid, *p*-methylbenzhydroxamic acid and *p*-toluenesulphonhydroxamic acid. With the exception of the last two, none of these produced signs at 150 mg./kg. The remaining two produced no signs at 100 mg./kg.

Signs of oxime poisoning always consisted of marked lethargy followed by prostration, muscular tremors, and loss of reflexes. The onset of signs occurred within 15 min. from the time of injection, and in those animals which survived recovery began within approximately one hour. After glyoxime the onset was delayed for several hours. Most of the oximes and all the hydroxamic acids examined were non-lethal to rats at 150 mg./kg. There were, however, notable exceptions to this. Thus di*iso*nitrosoacetone and *p*-methoxy*iso*nitrosoacetophenone produced deaths at a dose of 30 mg./kg., whereas benzhydrazide, glyoxime, *iso*nitrosoacetylacetone, mono*iso*nitrosoacetone and phenylglyoxime proved lethal at 100 mg./kg.

Screening of Oximes and Hydroxamic Acids Against Sarin Poisoning in Rats.—The dose of sarin used in these experiments (2 × LD50) when given alone always killed all the rats.

The oximes, when tested "prophylactically" and "therapeutically," fell into three distinct groups. The eight oximes comprising Group I proved highly effective against sarin poisoning, saving the lives of at least 80% of all animals employed in the tests. The five oximes of Group II, although exhibiting signs of biological activity in that there was always subtotal mortality in one of the two tests, appeared, however, to be of little practical interest. The remaining ten oximes were almost completely inactive. The oximes in the particular groups were as follows:

Group I.—Diacetylmonoxime, glyoxime, *iso*nitrosodiethylketone, mono*iso*nitrosoacetone, 2-methyl-3-oximino-4-oxopentane, 2-oxo-3-oximinopentane, salicylaldoxime and tri*iso*nitrosopropane.

Group II.—*Iso*nitrosoacetylacetone, α-furfuraldoxime, gallacetophenone oxime, pyrogallaldoxime and *iso*nitrosoacetophenone.

TABLE I

DOSES OF OXIMES PRODUCING NO SIGNS OF POISONING IN RATS

Oxime given by i.p. injection. Maximum dose, 150 mg./kg.

Dose (mg./kg.)	150	100	67	45	30	20	13
Oxime	Acetoxime Diacetyl monoxime Dihydroximinopyrrolidine α-Furfuraldoxime Pyridine-3-aldoxime methiodide Tri*iso*nitrosopropane	Pyridine-2-aldoxime methiodide Pyrogallaldoxime	Benzhydrazide Gallacetophenone oxime Glyoxime *Iso*nitrosodiethylketone Phthaloylhydroxylamine Salicylaldoxime	2-Oxo-3-oximinopentane	*Iso*nitrosoacetylacetone *Iso*nitrosoacetophenone Mono*iso*nitrosoacetone 2-Methyl-3-oximino-4-oxopentane Pyridine-4-aldoxime methiodide	Phenylglyoxime	Di*iso*nitrosoacetone *p*-Methoxy*iso*nitrosoacetophenone

Group III.—Acetoxime, benzhydrazide, di*iso*nitrosoacetone, dihydroximinopyrrolidine, *p*-methoxy*iso*nitrosoacetophenone, phenylglyoxime, phthaloylhydroxylamine, pyridine-2-aldoxime methiodide, pyridine-3-aldoxime methiodide and pyridine-4-aldoxime methiodide.

None of the hydroxamic acids was effective at the maximum dose used in the screening tests.

Of the eight oximes in Group I, four, namely mono*iso*nitrosoacetone (MINA), diacetylmonoxime (DAM), 2-oxo-3-oximinopentane, and 2-methyl-3-oximino-4-oxopentane, have the same general formula I, $R=CH_3CO$, and $R'=H$, Me, Et and *i*Pr respectively. A fifth oxime *iso*nitrosodiethylketone (I, $R=C_2H_5CO$, $R'=CH_3$) is isomeric with 2-oxo-3-oximinopentane.

The Toxicity of Sarin to Rats.—Each day when the LD50 of sarin in the presence of an oxime was determined, the LD50 of the inhibitor alone was checked on a control group of 20 rats, 4 animals being used at each dose level. Throughout the course of this work there were twelve tests in all, comprising a total of 240 rats.

A statistical analysis of the variations between tests was made following Finney (1947), and Table II gives the analysis of variance.

TABLE II

ANALYSIS OF VARIANCE OF MORTALITY PROBITS FOR THE SARIN CONTROL TESTS

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square
Regression	1	79·826	
Deviations from linearity ..	3	0·559	0·185
Between doses	4	80·385	
,, tests at each dose ..	55	38·484	0·700
Total	59	118·869	

Neither the sum of squares due to deviations from linearity nor that due to the difference between tests was significant at $P=0.05$ when tested as χ^2, nor do the two mean squares differ significantly (Fisher's F test). Thus the observations could be combined to provide an estimate of the sarin probit-regression line, from which the LD50 for sarin was estimated as 0.127 mg./kg. with 95% fiducial limits of 0.121 mg./kg. to 0.132 mg./kg. This value for the LD50 of sarin has been used throughout the paper for calculation of the efficiency of oximes to rats and agrees closely with the values 0.123 and 0.124 mg./kg. obtained earlier at this establishment.

Comparison of the Effectiveness of Five Similar Oximes Against Sarin Poisoning in the Rat.—MINA, DAM, 2-methyl-3-oximino-4-oxopentane, 2-oxo-3-oximinopentane and *iso*nitrosodiethylketone have been compared on a molar basis for their protective effect against sarin poisoning in the rat. The sarin was given by subcutaneous injection 15 min. after the oxime and the LD50 compared with the LD50 for sarin alone.

The results (Table III) show that these five oximes when given under comparable conditions exert a similar degree of protection against sarin

TABLE III

PROTECTIVE EFFECT OF EQUIMOLAR DOSES OF 5 SIMILAR OXIMES AGAINST SARIN POISONING IN THE RAT

Sarin, s.c. injection in 1 ml. saline/kg. Oxime, i.p. injection in 5 ml. water/kg., 15 min. before sarin

Oxime	Dose (mg./kg.)	A LD50 Sarin (mg./kg.) Limits $P=0.05$	A/LD50 Sarin
—	—	0·127 (0·121–0·132)	1
1. MINA	35	0·58 (0·46 –0·74)	4·6
2. DAM	41	0·71 (0·49 –1·02)	5·6
3. 2-Methyl-3-oximino-4-oxopentane	52	0·58 (0·46 –0·74)	4·6
4. 2-Oxo-3-oximinopentane	46	0·63 (0·41 –0·96)	5·0
5. *Iso*nitrosodiethylketone	46	0·43 (0·36 –0·50)	3·4

poisoning. Two, namely MINA and DAM, have therefore been studied in further detail. MINA, one of the more toxic of the oximes, is both a good reactor with sarin and a good reactivator of sarin-inhibited ChE. DAM, however, although considerably less toxic than MINA is neither a good reactor nor reactivator. These two oximes were chosen in order to try to elucidate more fully the possible mechanism of action of oximes *in vivo*. Experiments were carried out to determine the LD50 values of different antiChE compounds with the administration of these two oximes under a wide variety of circumstances. These involved variations in species used, in dosage of oxime and in time intervals between the administration of oxime and inhibitor.

Effect of Increase of Dose on the Protective Effect of DAM Against Sarin Poisoning in the Rat.—Since DAM produced no signs of oxime poisoning at the maximum dose of 150 mg./kg. used in the screening test, further toxicity studies were carried out. At a dose of 300 mg./kg. in the rat, DAM produces marked lethargy with a tendency towards prostration. At doses above this, complete prostration with partial or total loss of the righting reflex occurs. The highest dose so far given has been 450 mg./kg., and, although the effects are severe, recovery begins within about 45 min. In experiments using sarin, the maximum dose of DAM has therefore been 250 mg./kg. Table IV

TABLE IV

EFFECT OF INCREASING DOSES OF DAM UPON THE LD50 OF SARIN IN RATS

Sarin, s.c. injection in 1 ml. saline/kg. DAM, i.p. injection in 5 ml. water/kg., 15 min. before sarin

DAM (mg./kg.)	A LD50 Sarin (mg./kg.) Limits $P=0.05$	A/LD50 Sarin
0	0·127 (0·121– 0·132)	1
41	0·71 (0·49 – 1·02)	5·6
150	3·37 (2·46 – 5·24)	26·5
250	7·48 (5·88 –10·51)	58·0

shows the degree by which the LD50 of sarin is raised in rats by three different dose levels of DAM given 15 min. before the sarin.

The degree of protection exerted by DAM in sarin poisoning increases with an increase in dose of the oxime. When 150 mg./kg. of DAM is given 15 min. before the sarin, up to 6 × LD50 of sarin may be given without any signs of antiChE poisoning becoming apparent. Owing to its inherent toxicity, the maximum dose of MINA which may be given without producing oxime poisoning is 35 mg./kg. With this dose and under the same conditions as above, the LD50 of sarin is raised 5–6 times.

The Protective Effect of DAM and MINA Given at Varying Intervals of Time after Sarin in the Rat. —Both DAM (150 mg./kg.) and MINA (35 mg./kg.) have been given to rats 30 sec. after the injection of sarin. MINA exerted a similar degree of protection as when given prophylactically, i.e., the LD50 of sarin was raised 5–6 times. On the other hand, DAM raised the LD50 of sarin approximately 14 times as compared with the 27-fold increase obtained when it was given 15 min. before the inhibitor.

It was decided that both these compounds should be submitted to a more critical test of their therapeutic potentialities. In preliminary experiments, equivalent doses of DAM and MINA (41 mg./kg. and 35 mg./kg. respectively) were given both 3 and 6 min. after 0.15 mg./kg. sarin which is approximately the LD80 of sarin. Groups of 12 rats were used for each test and the results were compared with a further group of animals receiving sarin only. When given 3 min. after sarin no signs of antiChE poisoning occurred in either group of rats tested, although in the control group receiving 0.15 mg./kg. sarin only, seven of the 12 animals tested died within 45 min. When the oximes were given 6 min. after the sarin most rats showed signs of antiChE poisoning at the time of injection of the DAM or MINA. Although two of the 12 animals receiving 41 mg./kg. of DAM died immediately following the injection of oxime, no further deaths occurred; 24 hours later eight animals appeared quite healthy, but the remaining two were rather weak. None of the 12 animals given 35 mg./kg. MINA died, and, 24 hours after the injection, all animals in this group appeared quite healthy. Of the control group, 9 of the 12 rats died within 55 min. No further deaths occurred after this time.

Following the results obtained in this experiment, further tests were carried out in which the oximes were not given until sarin poisoning had developed. Two dose levels of sarin were used, 0.16 mg./kg. (approximately the LD90) and 0.24 mg./kg. (2 × LD50). The time of onset of signs was taken as the time when muscular fasciculations and mild convulsions first occurred. The results for 0.24 mg./kg. sarin are given in Table V.

TABLE V

EFFECTIVENESS OF DAM AND MINA WHEN GIVEN AFTER THE ONSET OF SARIN EFFECTS IN RATS

Sarin, 0.24 mg./kg. (2 × LD50) by s.c. injection in 1 ml. saline/kg. DAM (41 mg./kg.) or MINA (35 mg./kg.) by i.p. injection in 5 ml. water/kg. at time of onset of sarin poisoning

	Mean Time to Onset of Sarin Poisoning (min.)	Mortality (24 Hr.)	Average Intensity of Sarin Poisoning 3 Hr. after Injection of Oxime	Av. % Loss in Body Wt. in 24 Hr.	General Condition of Animals after 24 Hr.
Sarin	3½	12/12	—	—	—
Sarin + DAM	3½	3/12	Moderate to severe	13	1 animal fairly normal, remainder weak
Sarin + MINA	3½	3/12	No signs of sarin poisoning	2	All animals normal

In the experiment using 0.16 mg./kg. sarin the results paralleled those given in Table V. In the control group of animals receiving sarin only seven of ten animals died within 15 min. In the group receiving 41 mg./kg. DAM after the onset of sarin effects, one animal died 9 min. after the initial injection of sarin and one died 24 hours after the injection. Of the remaining 8 animals 7 had shown a marked loss in body weight 24 hours after the injections and were in a weak condition. None of the group of the animals given 35 mg./kg. of MINA died and all appeared quite healthy after 24 hours.

Although in both series of tests DAM and MINA at equivalent doses appeared almost equally effective in saving life, there was a marked difference in the signs of sarin poisoning between the two groups, following the injection of the oximes. With MINA, convulsions ceased shortly after the

injection of MINA and the animals rapidly became prostrate. Within one hour, although the animals appeared rather weak, all signs of antiChE poisoning had disappeared—i.e., generalized fasciculations and convulsions were no longer observed. Three hours after the initial injections, all surviving animals appeared normal. Although 9 of 12 animals given 41 mg./kg. of DAM survived a dose of sarin equivalent to twice the LD50, these animals still showed moderate to severe effects four hours after the injections with almost continuous convulsive spasms in all but one. Twenty-four hours later the animals were very weak and showed an average loss in weight from that of the previous day of approximately 13%. The marked loss in weight is an indication of the poor condition of the rats and is probably due to dehydration caused by the excessive salivation and micturition following marked sarin effects, with an inability to eat or drink because of the ensuing weakness.

Effect of DAM and MINA Against Sarin Poisoning in Species Other Than the Rat.—DAM (150 mg./kg. i.p.) has been tested prophylactically against sarin poisoning in mice, guinea-pigs, rabbits and monkeys. This dose of DAM never caused any signs of oxime poisoning. In each of these species, DAM was about ten times less effective than in the rat. Table VI summarizes the results, the figure for rats being given for comparison.

TABLE VI

PROTECTIVE EFFECT OF DAM AGAINST SARIN POISONING IN DIFFERENT SPECIES

Sarin, i.v. injection in monkeys, s.c. injection in all other species
DAM, 150 mg./kg. by i.p. injection, 15 min. before sarin

Species	A LD50 Sarin (mg./kg.) Limits $P=0.05$	B LD50 Sarin (mg./kg.) with DAM Limits $P=0.05$	B/LD50 Sarin
Rat ..	0·127 (0·121–0·132)	3·37 (2·46 –5·24)	26·5
Mouse ..	0·22 (0·19 –0·26)	0·38 (0·32 –0·45)	1·7
Guinea-pig	0·054 (0·048–0·060)	0·14 (0·11 –0·16)	2·
Rabbit ..	0·044 (0·037–0·052)	0·071 (0·049–0·120)	1·6
Monkey*	Approx. 0·025	Approx. 0·08	Approx. 3·0

* One determination only.

In toxicity tests on other species, MINA was lethal to mice and rabbits at 150 mg./kg. Guinea-pigs were an exception, in that no signs occurred. When 150 mg./kg. of MINA was given to guinea-pigs, 15 min. before sarin, the LD50 was raised approximately four times, a degree of protection similar to that exerted by the same dose of DAM. At 35 mg./kg., MINA gave no significant protection. In view of its toxicity, MINA has not yet been tested against sarin poisoning in any other species.

Effect of DAM and MINA Against Other AntiChE Compounds in Rats.—DAM (200 mg./kg. i.p.) has been examined prophylactically in rats for its effect against TEPP and dyflos. When given 15 min. before the antiChE, the LD50 of TEPP was raised almost twice. When tested against dyflos, 200 mg./kg. of DAM raised the LD50 approximately 1.3 times. MINA (35 mg./kg. i.p.) was also tested against TEPP, but raised the LD50 only 1.2 times.

Determination of the Length of Time after Injection for which DAM Retains its Protective Effect Against Sarin Poisoning in the Rat.—An experiment was carried out to determine the length of time for which a standard dose of 150 mg./kg. of DAM would maintain its effectiveness after injection in the rat. The DAM was given to rats at varying intervals before the subcutaneous administration of sarin (0.5 mg./kg., i.e. $4 \times$ LD50). When given alone this dose of sarin produced death within 3 min. From Table VII it can be seen that 150 mg./kg. of DAM affords complete protection against $4 \times$ LD50 of sarin for 95 min. after injection and for a period of 55 min. almost completely suppresses sarin effects.

TABLE VII

ESTIMATION OF THE PERIOD FOR WHICH DAM RETAINS ITS PROTECTIVE EFFECT AGAINST SARIN POISONING IN RATS

Sarin, 0.5 mg./kg. ($4 \times$ LD50) by s.c. injection in 1 ml. saline/kg.
DAM, 150 mg./kg. by i.p. injection in 5 ml. water/kg.

Time Interval between DAM and Sarin (min.)	No. Rats/ Group	Number of Animals Showing:			
		No Signs or Slight Fasciculations	Slight or Moderate Sarin Effects	Severe Effects with Recovery	Death
Controls (no DAM)	3	—	—	—	3 (in 3 min.)
5	4	4	—	—	—
15	2	2	—	—	—
35	4	4	—	—	—
55	4	4	—	—	—
75	4	3	1	—	—
95	4	2	2	—	—
120	4	1	1	1	1 (in 6 min.)
150	3	1	—	1	1 (in 6 min.)

DISCUSSION

The oximes were more effective than the hydroxamic acids when tested *in vivo* for their protective effect against sarin poisoning in the rat. Oximes having the general formula I, $R=CH_3CO$ were the most active. Of this group DAM afforded the greatest degree of protection, since its low toxicity enabled larger doses to be given.

It has been shown *in vitro* that oximes both react with and reactivate ChE inhibited by

antiChE compounds. It is therefore probable that the protective effect of oximes *in vivo* can be related to either or both of these factors. When compared on a molar basis and given 15 min. before sarin to rats, DAM, MINA, 2-methyl-3-oximino-4-oxopentane, and 2-oxo-3-oximino-pentane are all equally effective in protecting against sarin poisoning. However, *in vitro*, their rates of reaction with sarin, and power to reactivate sarin-inhibited ChE, vary considerably. In Table VIII the rate constants for the reaction of this series of oximes and also of *iso*nitrosodiethyl-

TABLE VIII

RATE CONSTANTS FOR THE REACTION OF OXIMES WITH SARIN

Oxime	Degree by which LD50 Sarin Raised by Equimolar Doses	*k_2 (Litres $mol.^{-1}$ $min.^{-1}$) at 25° C., *p*H 7·4
MINA	4·6	28·0
DAM	5·6	5·2
2-Methyl-3-oximino-4-oxopentane	4·6	2·5
2-Oxo-3-oximinopentane	5·0	3·4
*Iso*nitrosodiethylketone	3·4	5·2

* Green and Saville (1956).

ketone with sarin (Green and Saville, 1956) are compared with the effectiveness of equimolar doses *in vivo*. There does not appear to be any direct correlation between the two.

A similar discrepancy is found when comparing rates of reactivation with *in vivo* activity. Thus MINA at a concentration of 10^{-2} M reactivates in 15 min. 90% of ChE fully inhibited by sarin (Childs *et al.*, 1955). The same concentration of DAM only shows 5% reactivation in 60 min. (Green, personal communication), yet DAM is equally as effective as MINA in the living animal when both are given before the sarin. Equally discrepant is 2-oxo-3-oximinopentane, which is as effective as MINA *in vivo* but has been found to be only a feeble reactivator (Green, personal communication).

When a comparison is made of the protective effect *in vivo* of MINA against sarin and TEPP poisoning respectively, the concentration of MINA in rats which will raise the LD50 of sarin almost five times raises the LD50 of TEPP only 1.2 times when the oxime is given 15 min. before the inhibitor. However, 10^{-2} M-MINA reactivates sarin-inhibited ChE 90% in 15 min., whereas TEPP-inhibited ChE is reactivated by the same concentration 53% in 15 min. (Childs *et al.*, 1955). Since only a small amount of active ChE is assumed to be necessary for normal function, the relatively small difference in the rate of reactivation by MINA of ChE inhibited by TEPP as compared with that inhibited by sarin would not appear to be sufficient to account for the relatively large difference in protective effect against the two inhibitors. However, it is known that there is a marked species difference in response to a single antiChE and similarly that the same species reacts differently towards different antiChE's (Frawley, Hagan, and Fitzhugh, 1952). This is probably related to the ease with which an inhibitor reaches the main sites of ChE activity. It is therefore to be expected that the efficacy of an oxime in antiChE poisoning will vary both in different species towards the same inhibitor, and in one species towards different inhibitors regardless of rate of reaction with the inhibitor and rate of reactivation of inhibited ChE. Pyridine-2-aldoxime methiodide is an example of a good reactor with sarin and reactivator of sarin-inhibited ChE. However, *in vivo* in the rat it is without effect against 2 × LD50 of sarin both when given 15 min. before or 30 sec. after the inhibitor. Kewitz and Wilson (1956), however, have shown that in mice pyridine-2-aldoxime methiodide exhibits quite a marked protective effect in paraoxon poisoning.

It is therefore clear that *in vitro* tests of rates of reaction and reactivation are not of themselves adequate and cannot take the place of an *in vivo* assessment of the effectiveness of these compounds.

The so-called " prophylactic " and " therapeutic " tests which have been employed throughout the main part of the work do not permit a clear interpretation of the mode of action of the oximes. Thus the " prophylactic " tests may lead to a situation in which the inhibitor is destroyed by reaction with the oxime before inactivation of the enzyme occurs. Since in the " therapeutic " tests no acetylcholine-like effects have occurred at the time of injection, it is impossible in these experiments to differentiate between reaction and reactivation as the mode of action of the oxime. In later experiments, however, where the oximes were not given until obvious sarin poisoning had occurred it was possible to distinguish between MINA and DAM, which when given " prophylactically " appeared equally effective. This type of experiment was carried out only in rats. Although DAM and MINA proved equally effective in saving life, MINA rapidly checked generalized fasciculations and convulsions so that in a relatively short time the animals appeared normal although rather weak. However, severe antiChE poisoning continued for several hours following the administration of DAM, and 24 hours later the animals were left exhausted and very weak. The

difference between the two groups was emphasized by the marked loss of weight which occurred in those animals given DAM.

As signs of antiChE poisoning had occurred before the administration of either oxime, the difference in therapeutic efficiency between MINA and DAM must be related to their ability to reactivate sarin-inhibited ChE. MINA is one of the fastest reactivators of sarin-inhibited ChE, whereas DAM exhibits only about one-fifth the activity of MINA. This result therefore emphasizes the importance of reactivation in the therapeutic treatment of antiChE poisoning.

Although DAM and MINA are highly effective against sarin poisoning in the rat, they are about ten times less effective in all other species so far tested when given prophylactically. This does not appear to be correlated with a different degree of absorption following intraperitoneal injection. A comparison has been made in rats and rabbits between the blood level of oxime at the time of injection of the antiChE and the degree of protection obtained. For example, in rabbits when the concentration of DAM in the blood is roughly twice that in the rat, only one-quarter the degree of protection against sarin poisoning is obtained. There is thus a marked difference in the response of different species to the protective effect of the same oxime.

The oximes have in general been shown to be potential antidotes to antiChE poisoning. On the basis of the work described above, an efficient oxime would seem to require the basic structure of MINA, so modified, however, as to increase its speed of reaction with the inhibitor and its power to reactivate inhibited ChE, with at the same time a reduction in toxicity. Each of these properties can be found in individual oximes, but so far no single oxime has been found which exhibits all three properties.

Summary

1. Twenty-three oximes and nine hydroxamic acids have been tested as possible antidotes in antiChE poisoning. This has involved a preliminary study of the toxicity of these compounds to rats.

2. By screening tests against $2 \times LD50$ of sarin in rats, eight oximes have been found to be the most effective.

3. Four of these eight oximes having the general formula

$$\begin{array}{l} CH_3—C=O \\ \quad\quad\quad | \\ R—C=NOH \end{array}$$

exhibit a similar activity when given at equimolar concentrations to rats, 15 min. before sarin.

4. Two oximes, DAM and MINA, have been examined in more detail both in other species and against other antiChE's in order to try to elucidate more fully their mode of action.

5. DAM, because of its lesser toxicity, can be used at a higher dose and therefore appears to be more effective than MINA.

6. Both DAM and MINA have been shown to be active in sarin-poisoned rats even when given after the onset of antiChE poisoning.

7. Some theoretical implications of the results have been discussed.

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THE NATURE OF THE TOXICITY OF 2-OXO-OXIMES

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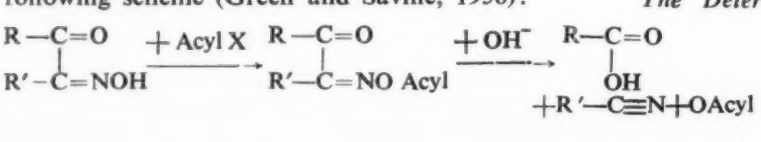
Some oximes have recently been tested as antidotes to organophosphorus anticholinesterase poisoning (Childs, Davies, Green, and Rutland, 1955; Holmes and Robins, 1955; Wilson and Ginsburg, 1955). Many of them are effective, particularly 1:2-dione monoximes of the type R.CO.C(R′):NOH where R is alkyl or aryl and R′ is H or alkyl. Those of greatest interest are mono*iso*nitrosoacetone (MINA; $R=CH_3$, $R'=H$) and diacetyl monoxime (DAM; $R=R'=CH_3$).

When tested therapeutically at equimolar doses, MINA and DAM proved equally effective in saving the lives of rats poisoned with *iso*propyl methylphosphonofluoridate (sarin). MINA, however, rapidly abolished acetylcholine-like effects, whereas, in those animals which had received DAM, convulsive spasms still persisted after several hours (Askew, 1956). However, along with other 2-oxo-aldoximes (R′=H), MINA is appreciably more toxic than either simple oximes or the related 2-oxo-ketoximes (R′=alkyl), so that its effectiveness is limited by the dose which may be given without toxic side effects. Hence it seemed desirable to determine how 2-oxo-aldoximes are more toxic than other oximes.

When oximes are given to animals, the concentration in the blood decreases relatively rapidly, but very little unchanged drug can be recovered from the urine (Davies and Rutland, 1956, unpublished). Breakdown in the body might occur by hydrolysis to hydroxylamine, or by enzymatic reduction to an amine, but neither of these products would explain the high toxicity of 2-oxo-aldoximes.

Many oximes are known to react in neutral solution *in vitro* with acylating agents of the type Acyl X, where Acyl may also be sulphonyl or phosphoryl and X is an acid radical—e.g., acetate or halide. With 1:2-dione monoximes, the intermediate then breaks down according to the following scheme (Green and Saville, 1956):



When R′ is H as in 2-oxo-aldoximes such as MINA, hydrogen cyanide (HCN) would be formed, whereas when R′ is alkyl as in 2-oxo-ketoximes such as DAM, no free HCN would be obtained. Of all normal oximes it is only 2-oxo-aldoximes which could break down directly by the above reaction sequence to give free HCN.

Acylating and phosphorylating agents exist in the body, hence this type of breakdown of 2-oxo-aldoximes to give HCN may occur *in vivo*. This possibility has been examined and it has been shown that sufficient HCN is formed by the metabolism of MINA and DINA (di*iso*nitrosoacetone, HC(:NOH).CO.HC:NOH) to account for their toxicity.

METHODS

Analytical Procedures

The Determination of Cyanide.—The method of Aldridge (1945) was used for the estimation of plasma cyanide and thiocyanate. The method does not differentiate between them when both are present together, thus a determination upon the plasma of an animal poisoned with an oxime which would liberate HCN would include both cyanide and thiocyanate. A part of the latter would be that normally present, whilst the remainder would be derived by detoxication of the HCN arising from the metabolism of the oxime. No attempt has been made analytically to differentiate between the cyanide and thiocyanate, but the amount of HCN derived from an appropriate oxime has been calculated by subtracting the control HCNS level from the total cyanide (i.e., HCN and HCNS) expressed as HCNS, and then converting the difference to HCN.

Blank estimations for cyanide were carried out on all oxime preparations used, and, with the exception of DINA, no free cyanide was detected. This negative result also showed that the oxime itself did not interfere with the test for cyanide. The amount of free cyanide found in DINA preparations was small and was insufficient to account for the increase of cyanide found after the injection of DINA into various animals.

The Determination of Oxime.—The method is essentially that of Blom (1926) for hydroxylamine. It has been modified for use with oximes in small volumes of blood. The principle

depends upon the acid hydrolysis of the oxime to hydroxylamine, which is then oxidized with iodine to nitrous acid. The nitrous acid is used to diazotize sulphanilic acid, the diazonium salt then being coupled with α-naphthylamine to give a pink colour.

The detailed procedure was as follows: *Reagents*—(1) iodine: 1.3 g. was dissolved in 100 ml. glacial acetic acid; (2) sulphanilic acid: 1 g. was dissolved in 75 ml. of distilled H_2O followed by the addition of 25 ml. glacial acetic acid; (3) α-naphthylamine: 0.3 g. was boiled with 100 ml. of distilled H_2O, the solution was filtered and 25 ml. glacial acetic acid was added to 75 ml. filtrate.

Procedure.—10% Trichloroacetic acid (TCA) (0.4 ml.) was added to a mixture of blood (0.2 ml.) and water (0.2 ml.), which was then centrifuged. The supernatant fluid was separated and a sample (0.2 ml.) was added to 10% sulphuric acid (0.8 ml.) and water (2.0 ml.). After being heated on a steam bath for 30 min. the solution was cooled to below 30° C. and was treated with iodine (0.1 ml.) to convert the free hydroxylamine into nitrous acid. This solution was then used to diazotize sulphanilic acid (0.1 ml.) in aqueous 40% sodium acetate (1.5 ml.). After removal of excess iodine with decinormal sodium thiosulphate (0.1 ml.), α-naphthylamine (0.1 ml.) was added. The total volume was made up to 5 ml., and after 10 min. the colour was read on an EEL absorptiometer (604 filter).

Calibration curves were obtained by adding known quantities of oxime to blood and subjecting these to the above procedure at the same time as the unknowns. The method has been used to determine oxime concentrations over the range 20–800 μg./ml. whole blood.

In vivo *Experimental Procedures*

In experiments upon rats, albinos (400 g.) in groups of 6 were used. After treatment with oxime or cyanide they were killed by a blow on the head and drained of blood, which was collected in heparinized tubes. Control animals, to which no oxime or cyanide had been given, were sacrificed at the same time and in a similar manner.

Rabbits and dogs (anaesthetized with urethane and pentobarbitone respectively) were used in order to make simultaneous serial estimations of cyanide and oxime in the blood. Blood samples from a T-cannula inserted into the femoral artery were obtained before, and at intervals after, the injection of the oxime. Samples of 3 ml. were taken from the rabbits and 4 ml. from the dogs, each sample being divided into two for the separate estimation of cyanide and oxime.

The cyanide plus thiocyanate was estimated in plasma, since Aldridge (1945) showed that thiocyanate could not be estimated in whole blood. The oxime was usually estimated in whole blood, for it was found to be uniformly distributed between cells and plasma.

Results

The signs of MINA or DINA poisoning are hyperventilation and muscular tremors, followed by prostration and loss of the righting reflex; respiration and heart beats then become progressively slower, culminating in death. These effects are similar to those observed in slow poisoning with cyanide, and in fact control groups of rats poisoned with 12 mg./kg. KCN s.c. could not be distinguished by direct observation from a group which had received a lethal dose of MINA (150 mg./kg. s.c.).

Qualitative confirmation of the formation of free HCN can be very easily obtained in MINA- or DINA-poisoned rats, for, when the gastro-intestinal tract is exposed, a very strong smell of HCN is at once apparent; it is also quite marked in the blood collected for analysis.

In some of the experiments on dogs, the expired air was passed through 10% caustic soda solution to absorb any HCN. The solution was analysed and approximately 8 μg. of HCN obtained in each of three successive 5-min. collection periods starting 15 min. after the intraperitoneal injection of MINA, 60 mg./kg.

The presence of free HCN in the plasma of rats poisoned with MINA (150 mg./kg. s.c.) was further demonstrated by the inhibitory effect of such plasma upon the oxidation of ascorbic acid

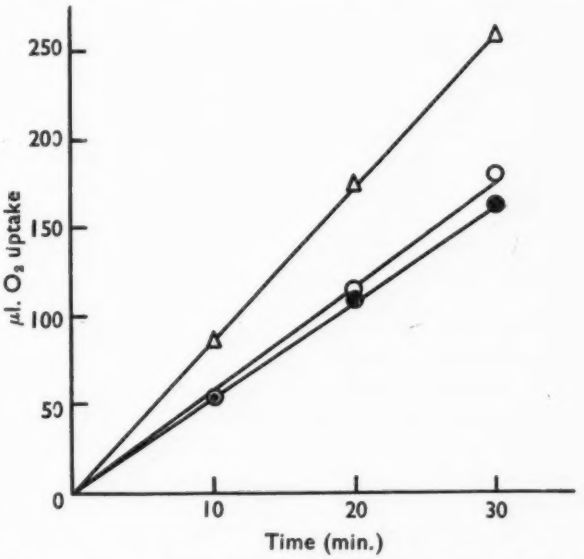


FIG. 1.—The inhibitory effect of plasma from rats poisoned with KCN (open circles) and MINA (filled circles) upon the oxidation of ascorbic acid by the cytochrome oxidase of rat heart. Control with normal plasma, triangles.

by a cytochrome oxidase preparation from normal rat heart (Schneider and Potter, 1943). This is shown in Fig. 1, which also includes for comparison the effect of plasma from KCN-poisoned rats (12 mg./kg. s.c.).

The Level of Cyanide in Oxime-poisoned Rats.—Separate groups of rats were given DINA, MINA, or DAM. Of two further groups, one was given KCN and the remaining served as untreated controls. Rats in each group were killed a few minutes before the previously determined expected time of death from the particular substance administered. Thus with MINA, after 150 mg./kg. s.c. the average time to death was 70 min., and these rats were therefore killed 50 min. after injection. Animals given KCN (12 mg./kg. s.c.), or DINA (25 mg./kg. s.c.), and which were expected to die about 25 min. after poisoning, were killed after 20 min. Since DAM is much less toxic, the time to death was not determined and, after a dose of 500 mg./kg. i.p., which caused acute effects, the animals were killed at an arbitrary interval of 20 min. The blood of each animal was collected separately and the plasma of each was analysed for "total cyanide"—cyanide plus thiocyanate. The results are shown in Table I.

TABLE I

THE CONCENTRATION OF CYANIDE IN THE PLASMA OF RATS POISONED WITH OXIMES OR KCN
(6 rats in each group)

	Dose (mg./kg. s.c.)	Mean HCNS+HCN	Mean HCN
		Expressed as μg. HCNS/ml. Plasma	HCNS+HCN−HCNS (Controls)×0·46 · μg. HCN/ml. Plasma
Controls ..		3·25 (2·77–3·76)	0
KCN ..	12	6·42 (6·04–6·75)	1·45
DINA ..	25	7·20 (6·66–7·87)	1·82
MINA ..	150	9·27 (8·50–9·95)	2·77
DAM ..	500 (i.p.)	3·16 (2·80–3·90)	0

If the cyanide in the plasma, following a killing dose of KCN, is taken as a criterion of cyanide lethality it is apparent from Table I that sufficient amounts can build up from these doses of DINA or MINA to account for their toxicity.

As mentioned in the introduction, DAM would not be expected to produce cyanide or thiocyanate directly, but would give rise to acetonitrile if it were broken down in the same way as the 2-oxo-aldoximes. However, aliphatic nitriles are metabolized to thiocyanate (Stoa, 1952), and it was found that a dose of 200 mg./kg. i.p. of acetonitrile (equivalent to 500 mg./kg. of DAM) caused a significant rise in total cyanide of the plasma of rats without producing marked effects. The rats were killed 20 min. after injection and the mean total cyanide (expressed as HCNS) of the plasma of 5 rats was 4.93 μg./ml. compared with the control level of 3.25 μg./ml. As may be seen from Table I, no increase in total cyanide was found 20 min. after the injection of 500 mg./kg. i.p. of DAM.

The Rate of Metabolism of Oximes in Rabbits and Dogs.—Simultaneous values of blood oxime and plasma cyanide were obtained after the intraperitoneal injection of 150 mg./kg. MINA to rabbits. The results showed a steady decrease in the concentration of oxime accompanied by a corresponding rise in plasma cyanide.

Similar experiments were carried out on dogs, not only because more frequent sampling was possible, but also to enable observations to be carried on for a longer period. The results with MINA and DAM are illustrated in Figs. 2 and 3, and show with MINA (Fig. 2) an inverse relationship between oxime and cyanide concentrations similar to that found in the rabbit. In experiments with DAM, despite a fall in oxime concentration and the presence of obvious toxic effects, no free cyanide was detected at any stage during the

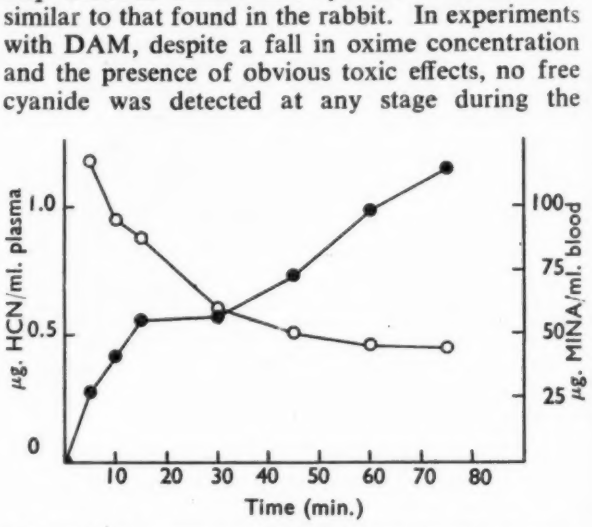


FIG. 2.—Blood concentrations of MINA (open circles) and cyanide (filled circles) in a dog given MINA, 60 mg./kg. i.p., at zero time.

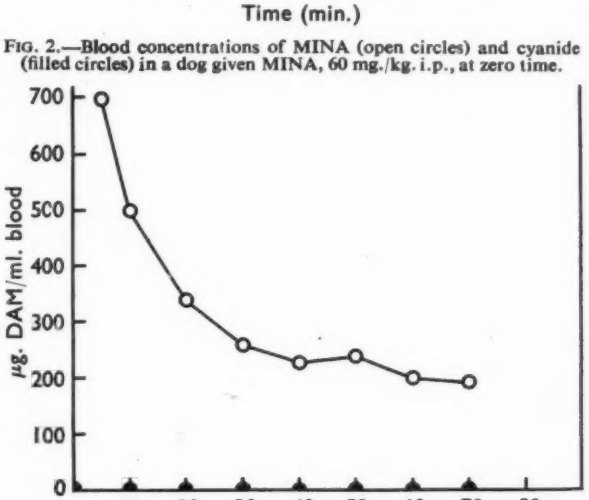


FIG. 3.—Blood concentrations of DAM (open circles) and cyanide (filled circles) in a dog given DAM, 500 mg./kg. i.p., at zero time. No free cyanide found.

experiment (Fig. 3). In one of the experiments on dogs, plasma was analysed for cyanide for up to 5 hr. after the administration of DAM (500 mg./kg. i.p.) and again no increase in total cyanide was found.

The Protection of MINA-poisoned Rats with Cyanide Prophylaxis.—If cyanide is actually produced in MINA poisoning and is responsible for the toxicity of this compound, known procedures which protect the animal against cyanide should be equally effective with MINA.

This was tested by giving MINA to groups of 4 rats, both alone or 10 min. after prophylaxis against cyanide. Prophylaxis consisted of either 22.5 mg./kg. sodium nitrite plus 1 g./kg. sodium thiosulphate, or 1 mg./kg. *p*-aminopropiophenone plus 1 g./kg. sodium thiosulphate, all given i.p. The MINA (150 mg./kg.) was generally given intraperitoneally, but in a few experiments was administered subcutaneously (200 mg./kg.) so that there was no possibility of direct reaction with prophylactic agent in the peritoneal cavity. With MINA alone, all rats showed severe effects and 7/8 died between 1–4 hr. With both forms of prophylaxis no toxic effects were seen and there were no deaths. In comparative prophylactic experiments with KCN (12 mg./kg. s.c.) no deaths or signs of cyanide poisoning occurred, although the KCN alone was always lethal.

Discussion

In the introduction, it has been suggested that the breakdown *in vivo* of DINA or MINA could lead to the formation of hydrogen cyanide, whereas by the same process DAM could not. Thus the relative toxicities of these substances should be related to the rate and extent of the formation of hydrogen cyanide. The toxicities of DINA, MINA, and DAM are compatible with this idea, the order is DINA>MINA⩾DAM. This is explicable since its structure suggests that 1 mole of DINA gives rise to 2 moles of HCN, whereas 1 mole of MINA produces only 1 mole of HCN, and DAM yields no free HCN. In addition, DINA is more unstable than MINA and appears to break down more quickly ; thus with a dose of DINA equivalent on a molar basis to one-quarter that of MINA used, two-thirds of the amount of cyanide produced by MINA was obtained from DINA (Table I).

The confirmation of this hypothesis depends upon the identification of cyanide in the tissues of animals poisoned with DINA or MINA. Quantitative estimations of total cyanide in the plasma of rats given lethal doses of DINA and MINA and for comparison KCN showed that more total cyanide was present following oxime poisoning than after KCN. As defined previously, estimations of total cyanide did not differentiate between thiocyanate and cyanide. However, the presence of free HCN has been unequivocally demonstrated. Thus in the experiments with cytochrome oxidase, it was shown that comparable amounts of HCN were present in the plasma from rats killed with MINA or KCN. Again, the existence of HCN, which can be detected by its odour in MINA poisoning, has been confirmed by its estimation in the expired air of MINA poisoned dogs. That cyanide has arisen directly from the breakdown of MINA is suggested by the inverse relationship of plasma cyanide and whole blood MINA concentrations found in rabbits and dogs.

Further evidence confirming the proposed mechanism of MINA toxicity has been obtained by the complete protection against lethal doses of MINA afforded by standard cyanide prophylaxis.

If DAM were broken down by the same process as MINA, acetonitrile would be produced, but no evidence of this mechanism has been obtained. The acute toxic effects of DAM appear to be due to the compound itself ; for example, an intravenous injection of DAM (150 mg./kg.) caused immediate cardiac arrest in rats and rabbits.

While it has been shown that the blood level of DAM falls fairly rapidly after injection, the fate of the substance is not known. However, from the experiments described, it does not appear to be metabolized by the same pathway as DINA and MINA.

Summary

1. The nature of the toxicity of the oximes mono*iso*nitrosoacetone (MINA), di*iso*nitrosoacetone (DINA), and diacetyl monoxime (DAM) has been investigated .

2. It has been shown that this is due, with MINA or DINA, to the formation of hydrogen cyanide.

3. There is no evidence to suggest that a similar mechanism will explain the toxicity of DAM.

The authors wish to thank F. A. B. Aldous and J. Bright for technical assistance.

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ON THE MODE OF ACTION OF TUBOCURARINE

BY

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It is commonly believed that the neuromuscular blocking action of (+)-tubocurarine cannot be satisfactorily explained if ascribed to its relatively weak inhibition of cholinesterases (ChE). An alternative explanation, however, has been suggested on the basis of the ability of tubocurarine to shift the optimum substrate concentration of acetyl-ChE to higher values (Župančič, 1953). The aim of the present work was to obtain more data on the action of tubocurarine on acetyl-ChE and to find out whether or not the results might throw any light on the behaviour of curarized muscles and ganglia.

METHODS

ChE activity was determined electrometrically by continuous titration with 0.02M-NaOH; the *p*H varied from 7.4 to 7.7. Solutions or suspensions were made up with saline I (mammalian tissues) or saline II (leech tissue). The volume of the reaction mixture was 10 ml.

Enzyme Preparations.—The human red blood cells in citrated blood were washed three times with NaCl (0.9%), haemolysed with distilled water, and diluted with the reaction mixture to 50 times the original blood volume.

The gastrocnemius muscles of cats and rabbits were cut twice to sections 25 μ thick, weighed, washed twice in a tenfold volume of saline I (0.1505M-NaCl, 0.0026M-KCl, 0.0018M-$CaCl_2$). A weight of 1 g. of normal muscle or 0.5 g. of denervated muscle was suspended in 10 ml. of the reaction mixture.

The grey matter of rabbit cerebral cortex was removed with scissors, weighed, finely ground and suspended in 9 parts of saline I. These preparations show predominantly acetyl-ChE activity (at pS=2 the hydrolysis of butyrylcholine was 18% of that of acetylcholine). One ml. of the suspension was used in 10 ml. of the reaction mixture.

The frozen skin-muscle bag of leech was cut twice to sections 25 μ thick, weighed, washed four times with saline II (0.1146M-NaCl, 0.0026M-KCl, 0.0018M-$CaCl_2$) whereby the butyryl-ChE of the interstitial fluid was removed. The sections (0.6 g.) were suspended in 10 ml. of the reaction mixture.

Substrate and Inhibitor.—Acetylcholine chloride was used as substrate throughout the experiments; (+)-tubocurarine chloride was used in a $10^{-3.5}$ M concn. and added to the reaction mixture before the substrate.

The measurements were performed at 38° C., except for the leech tissue, where the temperature was 18° C.

RESULTS

The inhibition by tubocurarine of all the cholinesterases used in this investigation is weak, and high concentrations must be used. However, a shift of the optimum substrate concentrations, toward higher values, was always found (Figs. 1–4).

DISCUSSION

In previous papers one of us (Župančič, 1953, 1955) proposed the hypothesis of receptor enzymes. According to this view, the receptors for various biologically active substances are assumed to be identical with more or less specific enzymes inactivating the particular substance. Thus the receptor protein for acetylcholine may be identical with tissue cholinesterase. Nevertheless it was felt that this view could hardly account for the behaviour of a family of agents of which tubocurarine is the classic representative; these substances are rather weak inhibitors of cholinesterase, but potent blocking agents of synaptic transmission. A closer examination, however, shows that this discrepancy is only superficial: from the pharmacological effects of such substances as stable choline esters, choline itself, nicotine or tetramethyl ammonium, it was concluded that acetylcholine acts at the moment when it is attached to the receptor cholinesterase and not at the moment of its hydrolysis. In neuromuscular transmission, acetylcholine thus causes quicker movements of ions across the post-synaptic membrane. The end-plate potential is thus set up while the cationic heads of its molecules are adsorbed to the anionic centres of receptor acetylcholinesterase. The hydrolysis by the esteroclastic centres only makes possible the repolarization of the membrane whereby the cycle can be repeated. The quaternary nitrogens of tubocurarine mole-

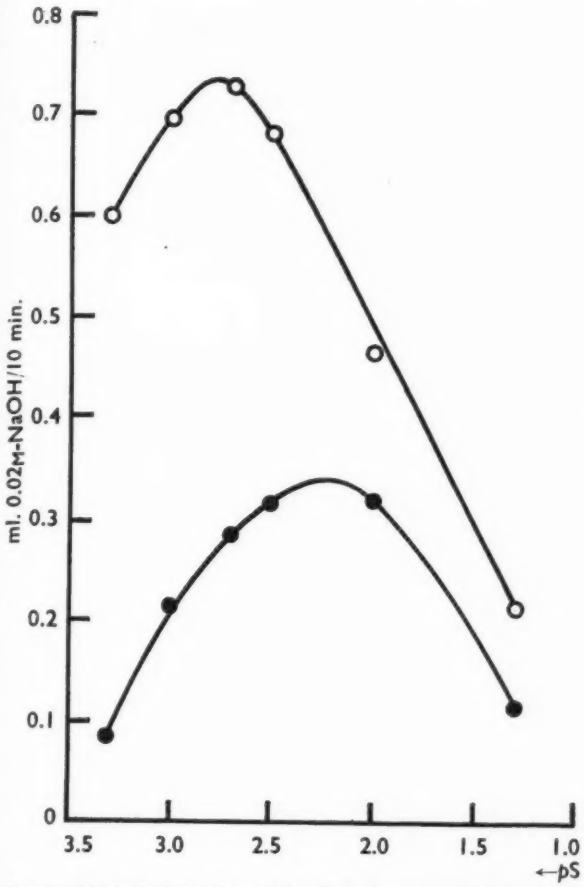

FIG. 1.—The hydrolysis of acetylcholine by human red blood cells in the presence (●) and absence (○) of $10^{-3.5}$ M-(+)-tubocurarine.

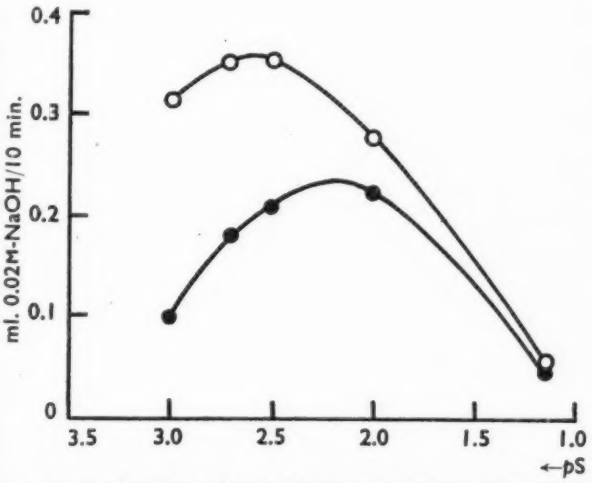

FIG. 2.—The hydrolysis of acetylcholine by cat gastrocnemius muscle in the presence (●) and absence (○) of $10^{-3.5}$ M-(+)-tubocurarine.

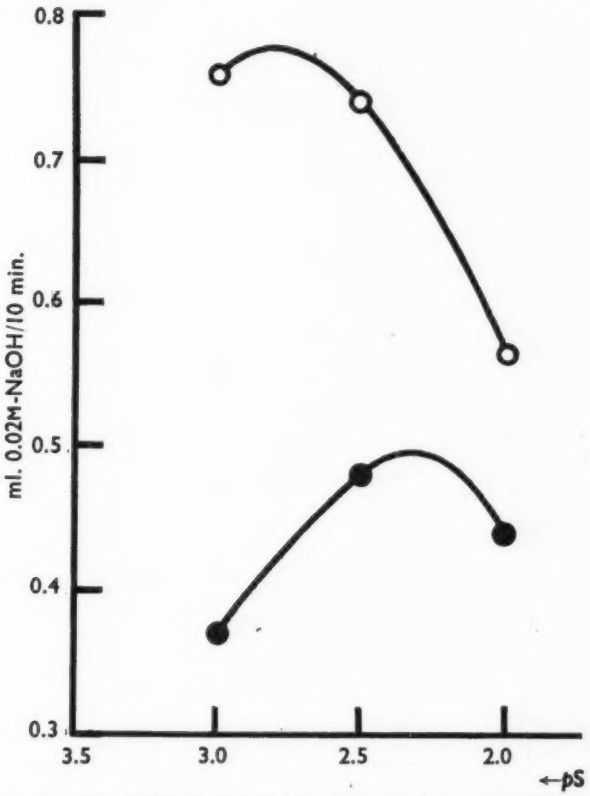

FIG. 3.—The hydrolysis of acetylcholine by rabbit cerebral cortex in the presence (●) and absence (○) of $10^{-3.5}$ M-(+)-tubocurarine.

cules thus compete with the cationic heads of acetylcholine for anionic centres of receptive cholinesterase without directly blocking the esteroclastic sites. Hence, with more and more anionic sites occupied by tubocurarine, the amplitude of the end-plate potential should decrease, and the whole process should eventually result in neuromuscular block as well as insensitivity to pharmacological acetylcholine. In the kinetics of acetylcholinesterase the theoretical effect of competition for anionic sites need not necessarily be inhibition of the hydrolysis, but rather prevention of inhibition by excess substrate—that is, a shift of the optimum substrate concentration to higher values (Augustinsson, 1948; Austin and Berry, 1953; Berry, 1953). With many substances even an activation of hydrolysis at higher substrate concentration has been shown. This is so, for example, with tetraethylammonium (Kensler and Elsner, 1951), a series of alcohols (Todrick, Fellowes, and Rutland, 1951), and with tubocurarine under certain conditions (Brzin and Župančič, 1955). In this laboratory a shift of the optimum substrate concentration accompanied by inhibition of activation of acetylcholinesterase was found for nicotine, hexa-

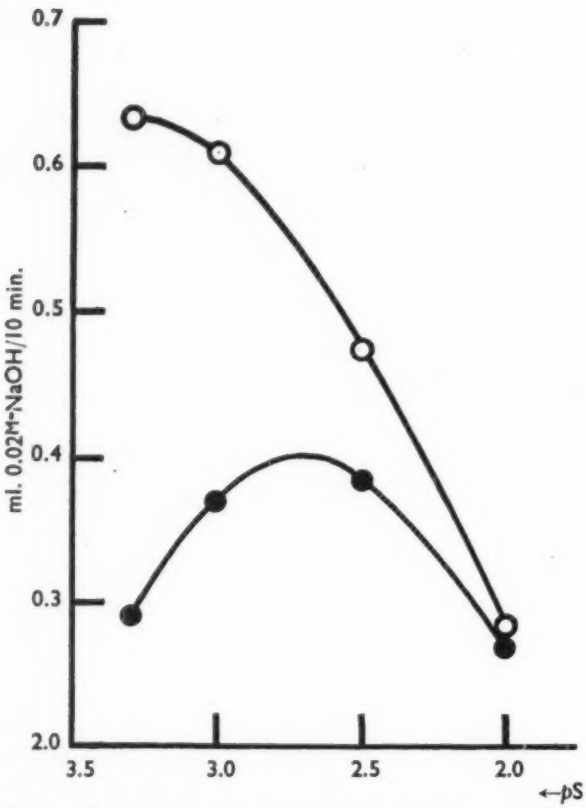

FIG. 4.—The hydrolysis of acetylcholine by leech skin-muscle bag in the presence (●) and absence (○) of $10^{-3.5}$ M-(+)-tubocurarine.

methonium, decamethonium and WIN 8077 (Kamarič and Župančič, 1956).

In our opinion, the so-called " direct " anticurare agents act by adsorption to the anionic sites of the receptive cholinesterase in the end-plate where they compete with tubocurarine.

On the other hand the " pure indirect " anticurare agents—such as dyflos or tetraethylpyrophosphate—acting through the esteroclastic sites of cholinesterase, do not shift the optimum substrate concentration (Augustinsson and Nachmansohn, 1949). It is clear that many substances can act at both the anionic and esteroclastic sites, and consequently display both the direct and indirect anticurare actions (e.g., neostigmine), the one or the other being prevalent.

With regard to the high concentration of tubocurarine used in this investigation the shift with curarization *in vivo* must be negligible. Yet it should be understood that the shift itself is not the cause of the synaptic block. It only represents evidence that tubocurarine combines with anionic sites of cholinesterase. In terms of the receptor-enzyme concept, the adsorption of a substance to the anionic centres of the receptive acetyl-cholinesterase logically calls for stimulation, or synaptic block, or both in succession.

SUMMARY

1. The influence of tubocurarine on the kinetics of 6 acetyl-cholinesterase preparations (human red blood cells, normal gastrocnemius muscle of cat, normal and denervated gastrocnemius muscle of rabbit, rabbit cerebral cortex, leech skin-muscle bag) was investigated.

2. Besides displaying a relatively weak inhibitory effect, tubocurarine produced a shift of the optimum substrate concentration towards higher values.

3. The significance of the results is discussed in terms of the receptor-enzyme concept.

We would like to thank Miss Berta Jenko and Miss Ljubica Rodič for valuable technical assistance.

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EFFECT OF NORADRENALINE ON URINE AND RENAL BLOOD FLOW

BY

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In six human subjects, noradrenaline infusions of 32–44 μg./min. produced albuminuria, and an average reduction of renal plasma flow, glomerular filtration rate, and urine flow to 43%, 62%, and 43% of control values respectively (King and Baldwin, 1956). The highest blood pressure recorded in these experiments was 204 mm. Hg systolic and 118 mm. Hg diastolic.

Despite the marked changes induced, this dose of noradrenaline is sometimes exceeded therapeutically; it is also much exceeded during the crises from a phaeochromocytoma. It thus becomes of interest to know the extent of the reduction in renal blood flow that may occur when noradrenaline is administered in doses sufficient either to restore the blood pressure in shock, or to raise it to levels comparable with those occurring during the crises from a phaeochromocytoma.

These problems have been investigated in dogs, because the necessary observations cannot be made in man in the presence of anuria or marked oliguria which necessitates measurement of renal blood flow by direct means. A résumé of this work was read to the Belgian Physiological Society on April 21, 1956 (Marson, 1956).

METHODS

Arterial blood pressure was recorded with a mercury manometer from the left femoral artery of dogs which were anaesthetized with chloralosane (110–120 mg./kg.); all measurements refer to systolic readings. Normal saline was infused at a rate constant for any one experiment into the left femoral vein. A freshly prepared solution of (±)-noradrenaline HCl was infused into the right femoral vein. The weights of noradrenaline are expressed in terms of the hydrochloride.

Study of Urine Flow.—The abdomen was opened and catheters were inserted into the lower ends of both ureters. The catheters led into small measuring cylinders which were changed every few minutes. After urine started flowing into the cylinders, and after preliminary observations, noradrenaline was infused intermittently and at various rates.

Study of Renal Blood Flow.—After homotransplantation of both kidneys into the neck, renal blood flow was measured by the methods of Brull (1931) and of Dor and Brull (1940) in which it is necessary to use a large perfusing dog and a small donor dog, both receiving artificial respiration by a positive pressure machine. The common carotid artery and external jugular vein of the perfusing dog were first ligated and divided, and Payr's cannulae were inserted into the clamped caudal ends of these vessels. Bilateral adrenalectomies were performed and both kidneys were then removed from the donor dog together with a few inches of aorta and inferior vena cava both above and below the renal vessels. The cephalic ends of the aorta and the inferior vena cava of the donor dog were then anastomosed to the caudal ends of the common carotid artery and external jugular vein of the perfusing dog. The arterial anastomosis is facilitated by a similarity in size between the common carotid artery of the large perfusing dog and the aorta of the small donor dog. The renal circulation had to be stopped for 3–4 min. during the transplantation. The free end of the aorta remained ligated, and the clamped free end of the inferior vena cava was attached to a special 10 or 20 ml. pipette. Renal blood flow was measured by releasing the clamp on the inferior vena cava proximal to the pipette and occluding the return of blood from the kidneys through the external jugular vein. The time for the passage of blood between two marks on the pipette was measured.

In some experiments, measured volumes of blood were removed through a cannula in the right femoral artery; this blood, to which heparin was added, was later reinjected into the left femoral vein.

RESULTS

Effect of Noradrenaline on Urine Flow.—Fig. 1 illustrates the alterations in urine flow and blood pressure during an experiment in which noradrenaline was given on seven occasions. The dose of noradrenaline was 1.65–22.4 μg./kg./min., and the infusions were continued for 2–18 min. The beginning of infusion was accompanied by an abrupt fall in the rate of urine flow proportional to the dose of noradrenaline; anuria occurred on the

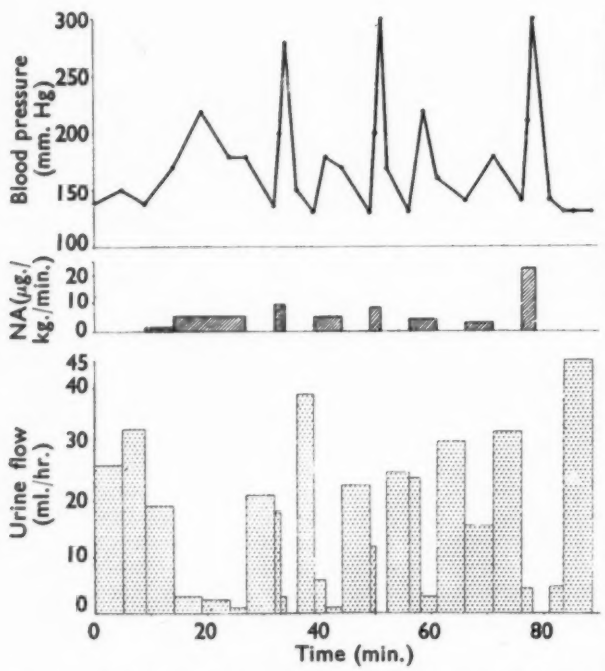

FIG. 1.—Dog, 14.9 kg., chloralosane 120 mg./kg. Showing the effect of intravenous noradrenaline (NA) on the urine flow and blood pressure.

three occasions on which the dose was 8.7 μg./kg./min. or higher.

Similar experiments were performed in twelve dogs. Noradrenaline always induced oliguria or anuria according to the dose. Table I shows the rates of noradrenaline infusion that produced anuria, together with the blood pressure at these times. The noradrenaline dose varied from 5.5–24.3 μg./kg./min.; the blood pressure was no higher than 200 mm. Hg in seven of the twelve experiments.

Anuria occurred within 15 sec. of the start of noradrenaline infusion. The urine flow always returned within 2 min. of stopping the infusion, often despite a marked temporary fall in blood pressure following withdrawal of the drug.

TABLE I

RATE OF NORADRENALINE INFUSION AT WHICH ANURIA OCCURRED

Wt. of Dog (kg.)	Noradrenaline (μg./kg./min.)	B.P. (mm. Hg)	
		Control	With Noradrenaline
10·3	7·45	165	200
12·4	12·4	150	200
3·5	13·2	130	200
14·9	15·4	180	230
10·0	16·2	120	240
7·5	8·6	165	190
13·3	24·3	160	210
12·0	9·6	135	220
16·7	5·5	185	190
14·9	8·7	180	300
9·1	6·1	185	200
24·9	10·5	160	180

Effect of Noradrenaline on the Blood Flow of Transplanted Kidneys.—Fig. 2 illustrates the changes in blood pressure and renal blood flow during six periods of noradrenaline infusion. In this experiment noradrenaline (1.4–3.7 μg./kg./min.) produced a prompt and marked fall in renal blood flow; when the noradrenaline dose was 4.6–13.8 μg./kg./min. the kidneys became very soft and pale, renal blood flow was completely arrested, and the blood pressure varied from 190 to 250 mm. Hg. On each occasion arrest of blood flow was observed for at least 2 min., but the flow always started again within half to one minute of stopping the noradrenaline. It was noticeable that the renal blood flow often recommenced 5–15 sec. before any fall in systemic blood pressure occurred, and that the rate of flow continued to increase despite a fall in blood pressure to below control levels.

Similar experiments were performed in five other dogs; Fig. 3 illustrates the changes in renal blood flow induced by various doses of noradrenaline. In any one experiment the degree of reduction in blood flow depended upon the dose of noradrenaline. Although the renal blood flow was not completely arrested in any of these further experiments, it was always reduced to below 14% of the control level. The graphs suggest that the rates of recovery of blood flow on stopping noradrenaline are partly dependent upon the duration of the noradrenaline infusion.

Alteration in Urine Flow Induced by Acute Haemorrhage and the Effect of Noradrenaline Before and After Reinjection of the Blood.—In one experiment (Fig. 4), removal of 450 ml. of blood (12.4 kg. dog) reduced the blood pressure from 180 to 100 mm. Hg. Anuria occurred at first and was followed by a slow urine flow. At this stage sufficient noradrenaline (8 μg./kg./min.) was given to restore the blood pressure to the original control level, and this was maintained for 20 min. Despite restoration of blood pressure, anuria persisted throughout the noradrenaline infusion. A slight urine flow returned 18 min. after stopping the infusion. After the reinjection of 200 ml. of blood a good diuresis occurred promptly, and was particularly marked for the first 5 min. A second 200 ml. blood produced a further diuresis. The dog having now received all but 50 ml. of the blood removed, noradrenaline was restarted. With an initial dose of 2 μg./kg./min. no reduction in urine flow occurred, but when the dose was raised

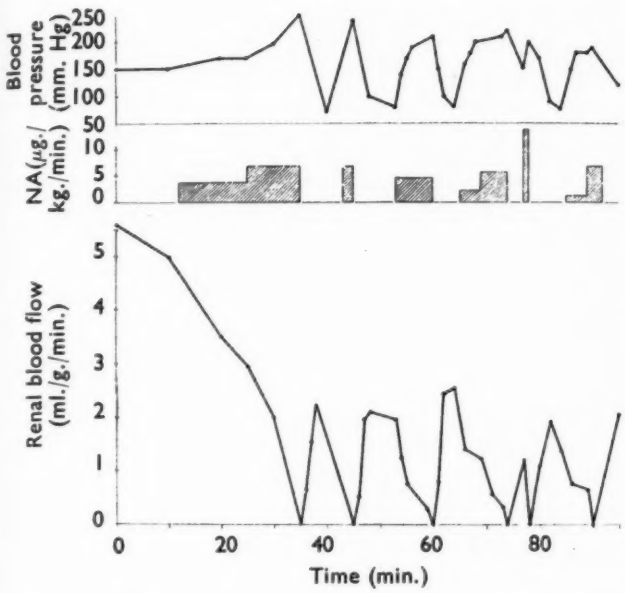

FIG. 2.—Dog, 26.7 kg., chloralosane 110 mg./kg. Showing the effect of intravenous noradrenaline (NA) on the blood flow of transplanted kidneys in the neck.

to 7.5 μg./kg./min. there was oliguria and the blood pressure rose above the control level. On stopping the noradrenaline there was a marked diuresis despite the fall in blood pressure to 100 mm. Hg, a level which, when induced by haemorrhage, had been accompanied by a great reduction in urine flow.

Similar experiments were carried out in four other dogs, and Table II shows the extent of the haemorrhage, its effect on blood pressure, and the dose of noradrenaline required to restore the blood pressure to the control level. Despite this correction, anuria occurred during the infusion in all but one dog in which oliguria was observed. A brisk and marked diuresis always occurred after stopping the noradrenaline and reinjecting the removed blood, this measure being accompanied by a return of the blood pressure either to the control level or slightly below it.

Effect of Noradrenaline on Renal Blood Flow During a Period of Acute Haemorrhage.—Fig. 5 illustrates the effect of acute haemorrhage on renal blood flow, removal of 225 ml. blood (13.5 kg. dog) having reduced the blood pressure from 115 to 35 mm. Hg with a spontaneous rise to 70 mm. Hg after 2 min. The renal blood flow fell from 2.1 to 0.6 ml./g./min. (29% of the con-

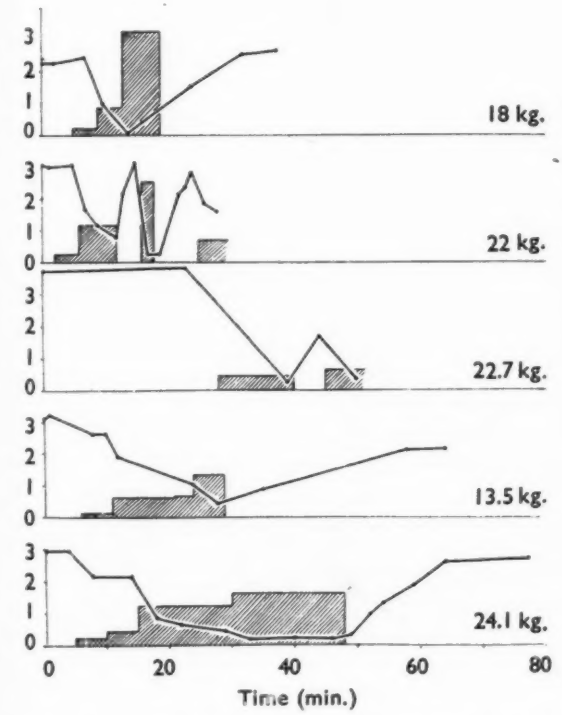

FIG. 3.—Five experiments showing the effect of intravenous noradrenaline (striped rectangles; × 10 μg./kg./min.) on renal blood flow (continuous lines; ml./g./min.). The dogs' weights are indicated.

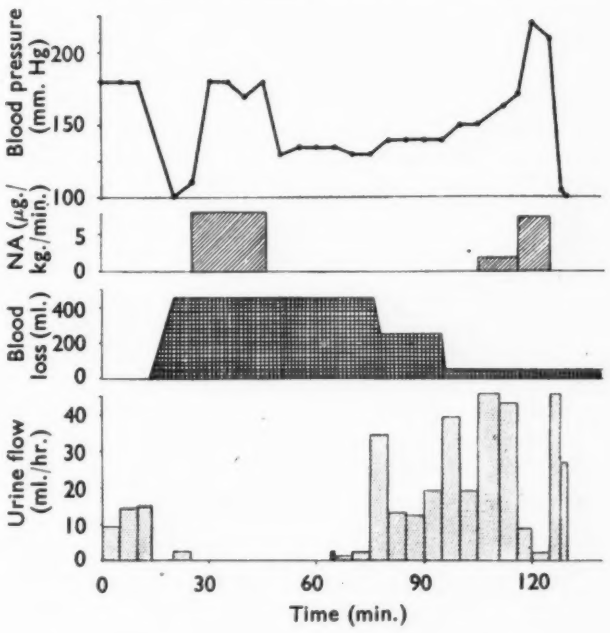

FIG. 4.—Dog, 12.4 kg., chloralosane 120 mg./kg. Showing the alteration in urine flow following acute haemorrhage, and the effect of noradrenaline (NA) before and after reinjection of the blood.

TABLE II

DOSE OF NORADRENALINE REQUIRED TO RESTORE BLOOD PRESSURE AFTER ACUTE HAEMORRHAGE

Anuria occurred in all but the fourth dog in which there was oliguria

Wt. of Dog (kg.)	Volume of Blood Removed (ml.)	B.P. (mm. Hg)		Noradrenaline (μg./kg./min.)
		Control	After Bleeding	
12·4	450	180	100	8·1
14·9	500	180	85	11·5
7·5	250	150	60	8·6
13·3	330	145	70	6·2
14·9	400	160	80	6·6

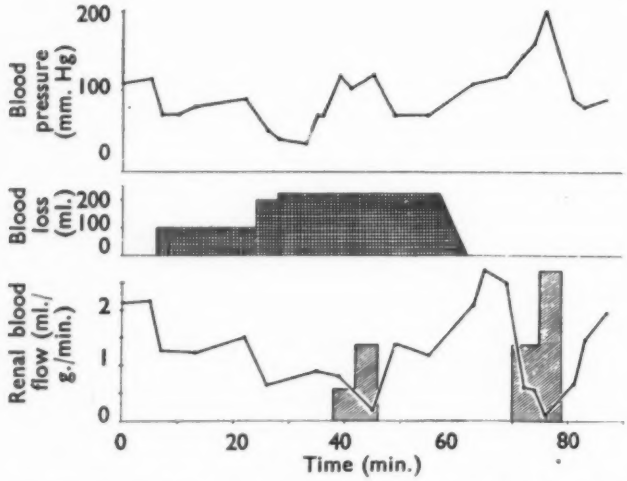

FIG. 5.—Dog, 13.5 kg., chloralosane 110 mg./kg. Showing the effect of intravenous noradrenaline (bottom section, striped rectangles; × 10 μg./kg./min.) on renal blood flow during haemorrhage and after restoration of the blood.

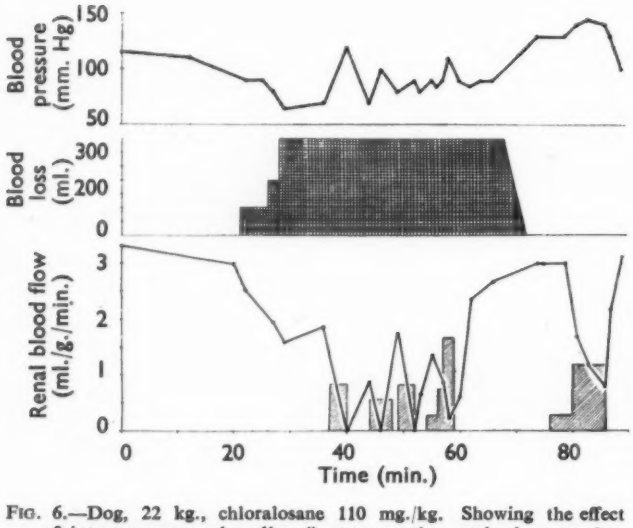

FIG. 6.—Dog, 22 kg., chloralosane 110 mg./kg. Showing the effect of intravenous noradrenaline (bottom section, striped rectangles; × 10 μg./kg./min.) on renal blood flow during acute haemorrhage and after restoration of the blood.

trol level). Noradrenaline (13.6 μg./kg./min.) then raised the blood pressure to 120 mm. Hg, but induced a further fall in renal blood flow to 0.19 ml./g./min. (9% of the control level). On stopping the noradrenaline and reinjecting the blood, the renal blood flow rapidly returned to the control level, but noradrenaline at the same rate again caused a marked reduction in renal blood flow. This reduction—to 0.6 ml./g./min.—was not so great as that before restoration of the blood, although further reduction in blood flow occurred on raising the noradrenaline dose.

Fig. 6 illustrates another similar experiment in which 350 ml. blood (22 kg. dog) was removed. Following this haemorrhage, the renal blood flow fell to approximately half the control level. Three separate infusions of noradrenaline (8.4, 5.6, and 8.4 μg./kg./min.) each caused complete arrest of renal blood flow, and simultaneously raised the blood pressure to 120, 100, and 90 mm. Hg respectively, the control levels before haemorrhage having been between 100 and 115 mm. Hg. A fourth infusion (up to 16.8 μg./kg./min.) greatly reduced the renal blood flow but did not arrest it. After stopping the infusion the blood pressure and renal blood flow were both appreciably higher than at the start of the blood loss, the recovery having occurred in 30 min. After restoration of the blood the effect of a further infusion of noradrenaline depressed the renal blood flow but not so much as before restoration.

Effect of Acute Haemorrhage, and of Noradrenaline, on Blood Flow and Urine Formation in Transplanted Kidneys.—Although only one experiment of this type was performed, the inclusion of observations on the rate of urine flow from the transplanted kidneys permitted a comprehensive study which incorporated many of the changes observed in the previous experiments. The results are illustrated in Fig. 7. The first infusion of noradrenaline was accompanied by a fall in renal blood flow from 3.68 to 1.81 ml./g./min. (49% of the control level), together with anuria. After removal of 560 ml. blood (18 kg. dog), the renal blood flow fell to 0.71 ml./g./min. (19.3% of the control level), and anuria occurred. During the blood loss, noradrenaline (9.2 μg./kg./min.) raised the blood pressure to the level preceding haemorrhage but decreased the renal blood flow still further to 0.18 ml./g./min. (4.8% of the control level). After stopping the noradrenaline and reinjecting the blood, the renal blood flow rose rapidly to 2.3 ml./g./min. and urine flowed

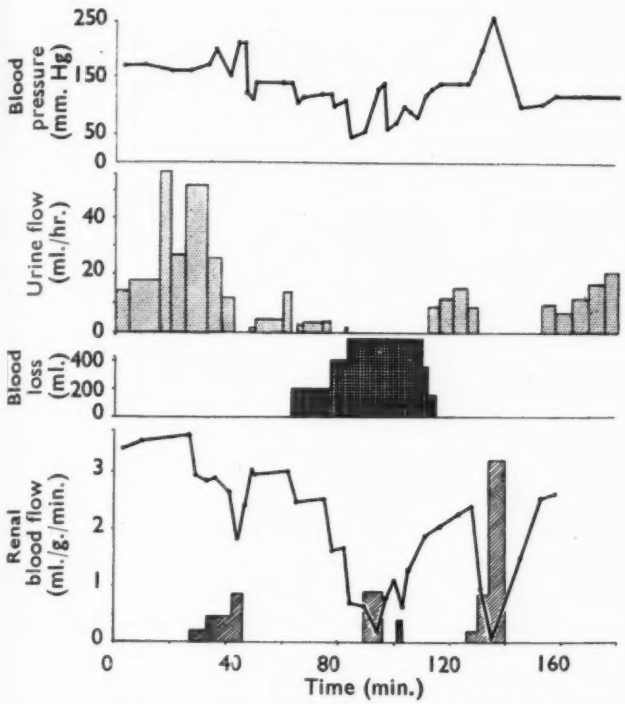

FIG. 7.—Dog, 18 kg., chloralosane 120 mg./kg. Showing the effect of acute haemorrhage on the blood flow and urine formation in transplanted kidneys, and the effects of noradrenaline (bottom section, striped rectangles; × 10 μg./kg./min.) before, during, and after the blood loss.

again. Further infusions of noradrenaline in varying doses showed that 2 μg./kg./min. had no definite effect on urine flow or renal blood flow, whereas 8.7 μg./kg./min. reduced renal blood flow to 1.0 ml./g./min. and stopped the flow of urine, and 32.3 μg./kg./min. reduced renal blood flow to 0.07 ml./g./min. On stopping the noradrenaline, the renal blood flow returned to 2.6 ml./g./min. after 13 min. and urine flow restarted.

DISCUSSION

The effects of noradrenaline on renal function have usually been studied by clearance techniques. These can only be used in the presence of a free urine flow, and they are more suited to experiments in which conditions remain constant, rather than to those in which marked variations in blood pressure, renal blood flow, and urine flow occur.

My experiments, in which the kidneys of one dog were transplanted into the neck of another dog, have permitted repeated direct measurements of renal blood flow irrespective of the urine flow. They have confirmed previous reports of reduction in renal blood flow with noradrenaline (man: Barnett, Blacket, Depoorter, Sanderson, and Wilson, 1950; Werkö, Bucht, Josephson, and Ek, 1951; King and Baldwin, 1956; dog: Pickford and Watt, 1951; Moyer and Handley, 1952). They have shown that the blood flow may be completely arrested; this arrest occurred abruptly on starting the noradrenaline infusion, and recovered rapidly on stopping the drug.

Urine flow decreased progressively with increasing doses of noradrenaline, and anuria was repeatedly observed. The blood pressure during the anuric periods was often only moderately raised and was rarely at those levels found in patients with a phaeochromocytoma. The extent to which renal blood flow falls during the hypertensive crises in such patients is not known, but albuminuria is commonly found (Howard and Barker, 1937; Fertig, Taylor, Corcoran, and Page, 1951), and anuria, oliguria, and azotaemia may occur (Labbé, Tinnel, and Doumer, 1922; MacKeith, 1944).

Moyer, Handley, and Huggins (1954), in studying the renal response to noradrenaline in dogs made hypotensive by repeated haemorrhage, reported that the restoration of normal blood pressure was accompanied by improvement in renal function provided the haemorrhage was not severe, but that, if severe, noradrenaline might cause a further reduction in the renal blood flow. Moyer, Morris, and Beazley (1955) studied the renal changes in man during severe haemorrhagic shock and after normal tension had been restored by noradrenaline. They found that noradrenaline might improve the renal function, but that complete restoration required an increase in the blood volume. In my experiments, hypotension induced by haemorrhage was accompanied by marked oliguria or anuria and by a considerable fall in the renal blood flow. On restoring the blood pressure to normal with noradrenaline, anuria persisted and the renal blood flow fell still further or, as in one dog, stopped completely. A rapid restoration of urine and renal blood flow followed the reinjection of blood.

Whereas all but one of the present studies on urine flow were performed on innervated kidneys *in situ*, the blood flow measurements were carried out on transplanted, and therefore denervated, kidneys. Denervation of the kidneys in dogs has been reported to increase the sensitivity to noradrenaline (Berne, Hoffman, Kagan, and Levy, 1952). In the one experiment in which I studied the urine flow of the transplanted kidneys the dose

of noradrenaline causing anuria was comparable with that having a similar effect in the innervated kidneys. This supports the observation of Pickford and Watt (1951) that denervation did not alter the sensitivity of the kidneys of the dog to noradrenaline.

Summary

1. In anaesthetized dogs urine flow was progressively reduced by increasing doses of (±)-noradrenaline. When anuria occurred, with sufficiently high dose, the systolic blood pressure was 200 mm. Hg or less in most dogs.

2. Noradrenaline produced an abrupt fall in, and sometimes complete arrest of, renal blood flow.

3. Reduction in urine and renal blood flow followed acute haemorrhage. Further reduction occurred when the blood pressure was restored with noradrenaline. Renal function rapidly recovered on reinjection of the removed blood.

4. Similar renal changes may possibly occur in patients during the crises from a phaeochromocytoma, or receiving noradrenaline in post-haemorrhagic shock.

This work was carried out at the Institut de Clinique et de Policlinique médicales, Université de Liège, during the tenure of a United Leeds Hospitals Travelling Fellowship. I am deeply grateful to Professor L. Brull under whose stimulating influence I worked.

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THE ACTION OF SOME NUCLEOTOXIC SUBSTANCES ON PREGNANCY

BY

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In a previous investigation (Didcock, Picard, and Robson, 1952) it was shown that podophyllotoxin, which is a nucleotoxic substance with striking inhibitory effects on certain tumours, will interrupt pregnancy in mice and rabbits. The relation between the dose necessary to affect pregnancy and that toxic and lethal to the mother—what might be called in this connexion the therapeutic ratio—was, however, low, being of the order of 4. It was of interest to investigate the effect of other nucleotoxic substances on pregnancy, to determine whether there is any relation between nucleotoxic activity and effect on pregnancy, and also whether there are any nucleotoxic substances with a higher therapeutic ratio than podophyllotoxin.

METHODS

The experiments were performed on mice and rabbits. In the former the duration of pregnancy was dated from the finding of the vaginal plug, and in the latter from the observed mating. In mice injected between the sixth and tenth days of pregnancy a laparotomy was performed to establish that a normal pregnancy was present. After this time it is possible to confirm the existence of pregnancy by abdominal palpation. Before the sixth day, when implantation occurs, the effect of the drug was established by dividing a number of mice, in which vaginal plugs had been found, into two groups. One group was treated and the other served as control.

The intra-amniotic and intraplacental injections were performed on rabbits under ether anaesthesia with full sterile precautions. The abdomen was opened by a midline incision and the uterus exposed. A particular site was chosen for injection and its various parts identified by transillumination from a strong source of light. The material was then injected in 0.1 ml. into the amniotic fluid or in 0.05 ml. into the placenta. For the latter injection it was desirable to have a needle fitted with a collar some 3–4 mm. from the tip to ensure that there should not be too deep a penetration and that the material was actually injected into the placenta.

The effect on pregnancy was determined by looking for vaginal bleeding or for presence of foetal remains in the cage, by changes in the maternal weight, by abdominal palpation, and by examination at laparotomy or post-mortem.

RESULTS

The Effects of Colchicine and Some of its Derivatives on Pregnancy

Colchicine.—Colchicine was injected subcutaneously in aqueous solution. The results are shown in Table I. On the day following the injection there was usually blood in the vagina of the mice, showing that a toxic effect on the foetus had started; occasionally abortion quickly followed, but sometimes there was a slower loss in maternal weight indicating gradual reabsorption of the foetuses. Of the two rabbits injected one was dead the next day and showed early haemorrhagic reabsorption, whereas the other died after two days, with no obvious effect on the foetus. The lowest dose to interrupt pregnancy consistently in mice is 2 mg./kg. which kills some of the mothers. The "therapeutic ratio" is therefore even lower than that of podophyllotoxin.

TABLE I

THE EFFECT OF COLCHICINE ON PREGNANCY

Animals	Duration of Pregnancy at Time of Injection (Days)	Dose of Colchicine (mg./kg.)	Interruption of Pregnancy*	Maternal Mortality
Mice ..	11	2	4/4	1/4
	10	1·5	2/5	1/5
	11	1	1/5	0/5
Rabbits ..	14	2	1/2	2/2

* Number of pregnant females in which abortion or foetal death occurred out of total number of females studied.

Demecolcin.—Demecolcin is an alkaloid which, like colchicine, is extracted from *Colchicum autumnale* and was first described by Santavy and Reichstein (1950). In animal experiments it is some thirty times less toxic than colchicine and has been used in the treatment of chronic myeloid leukaemia (Moeschlin, Meyer, and Lichtman, 1953). This derivative of colchicine was tested for

TABLE II
THE EFFECT OF DEMECOLCIN ON PREGNANCY

	Route of Administration	Dose (mg./kg.)	Interruption of Pregnancy	Maternal Mortality
Mice 11–13 days pregnant	Subcutaneous	1	4/4	0/4
	,,	2	4/6	0/6
	,,	4	5/5	0/5
	,,	8	3/3	0/3
	,,	16	4/4	1/4
	Oral	1	0/5	0/5
	,,	2	2/5	0/5
	,,	4	3/4	0/4
	,,	8	5/5	4/5
	,,	16	3/3	2/3
Rabbits 13–16 days pregnant	Subcutaneous	1	0/2	0/2
	,,	2	2/2	0/2
	,,	4	2/2	0/2
	,,	8	2/2	1/2
	Oral	2	0/2	0/2

its effect on pregnancy, and the results are shown in Table II. The effect on pregnancy was manifest in from 1–2 days after injection. The "therapeutic ratio" of demecolcin administered subcutaneously to mice and rabbits is about 4, but the drug is less effective when given orally.

Trimethyl Colchicinic Acid Methyl Ether.—Trimethyl colchicinic acid methyl ether (TME) was first prepared by Ullyot and has been tested for its ability to damage sarcoma 37 in mice (Leiter, Downing, Hartwell, and Shear, 1952). These workers found that TME has a therapeutic ratio—maximum tolerated dose to minimum effective dose—of 50 whereas colchicine, under similar conditions has a therapeutic ratio of 2. It was tested for its effect on pregnancy in mice and rabbits, and the results are shown in Table III. The effect on pregnancy came on more gradually than with podophyllotoxin or demecolcin, the characteristic effect being a gradual loss in weight and abortion or reabsorption in 2–3 days. TME has a "therapeutic ratio" of about 12 in mice since a dose of 1.5 mg./kg. of the tartrate, equivalent to 1 mg. of the alkaloid, will interrupt pregnancy in all animals without producing any maternal deaths whereas a dose of 18 mg./kg. of the tartrate kills some of the mothers.

In rabbits a dose of 4 mg./kg. will consistently interrupt pregnancy without producing toxic effects on the mother, whereas doubling the dose is lethal to the mother. The therapeutic ratio in this species is therefore about 2.

Since TME has been used orally in patients, its effect by this route was also investigated in animals; it was less effective and more toxic to the mothers than when given by injection.

The drug was also given before implantation, i.e., before the sixth day. Pregnancy occurred in 18 out of 45 of the mice which received a dose of 4 mg./kg., whereas 22 out of 42 control mice became pregnant. With 8 mg./kg. TME 2 out of 32 of the treated mice became pregnant against 14 of the 19 untreated mice. The "therapeutic ratio" is therefore much lower at this stage of pregnancy.

Local Action of Trimethyl Colchicinic Acid Methyl Ether and Podophyllotoxin.—It seemed likely that the effects of the colchicine derivatives on pregnancy were due to a selective toxic action on the foetus, the foetal tissue being more sensitive to the drugs than the maternal organism. The drugs were therefore applied directly to the intrauterine tissues by intra-amniotic or intraplacental injection.

The rabbits were injected between the 15th and 21st days of pregnancy and laparotomies were performed 5–8 days after injection. The results are shown in Table IV. It was found that the injection of a control solution into either the amniotic fluid or the placenta produced no effect on the

TABLE III
THE EFFECT OF TRIMETHYL COLCHICINIC ACID METHYL ETHER (TME) ON PREGNANCY

Animal	Duration of Pregnancy (Days)	Route of Administration	Dose of TME* (mg./kg.)	Interruption of Pregnancy	Maternal Mortality
Mice	13	Subcutaneous	1·5	7/7	0/7
	14	,,	4	14/14	0/14
	14	,,	8	7/7	0/7
	13	,,	18	4/4	3/4
	12	Oral	2	3/5	0/5
	12	,,	4	6/9	0/9
	12	,,	8	4/4	3/4
	6	Subcutaneous	4	10/10	1/10
	6	,,	8	8/8	2/8
	7	,,	4	5/5	0/5
	8	,,	4	5/5	0/5
	9	,,	4	6/6	0/6
	15	,,	1·5	0/3	0/3
	15	,,	4	14/14	0/14
Rabbits	15	,,	1	0/2	0/2
	13	,,	2	2/4	0/4
	14	,,	4	5/5	0/5
	15	,,	8	1/1	1/1

* Tartrate used. The actual dose of alkaloid, therefore, is about two-thirds the stated dose.

TABLE IV
THE EFFECT ON PREGNANCY OF TRIMETHYL COLCHICINIC ACID METHYL ETHER AND PODOPHYLLOTOXIN INJECTED INTO THE AMNIOTIC FLUID OR PLACENTA IN RABBITS

The first numeral shows the total number of sites in which the pregnancy was interrupted; the second shows the total number of sites injected with that particular dose

Dose of TME (μg.)	5	10	25	50	100	200	500	1,000
Intra-amniotic ..	0/1	3/5	7/9	5/5				
Intraplacental ..			0/3	0/5	1/4	1/3	2/2	1/1
Dose of podophyllotoxin (μg.)	**0·2**	**1**	**2**	**5**	**10**	**20**	**100**	
Intra-amniotic ..	0/3	0/5	0/6	4/8		3/3	2/2	
Intraplacental ..				0/2		2/9	3/6	

foetus. When pregnancy was interrupted following intra-amniotic injection, a small dead foetus was usually found, but at a few sites a reddish brown gelatinous mass was all that remained of the conceptus. The following examples illustrate these effects. The uterus of a rabbit 21 days pregnant contained 11 foetuses. Four of these had 25 μg. or 50 μg. TME injected into the amniotic fluid, the remaining 7 serving as controls. The mother was killed 5 days after injection and the uterine contents removed and examined. All the untreated foetuses were alive and appeared normal. The weights of these seven foetuses varied from 16 to 20 g. and the weights of the placentae from 2.3 to 4 g. Where the TME had been injected into the amniotic fluid all the foetuses were dead. The weights of the dead foetuses ranged from 4–6 g., the corresponding placentae weighing from 1–3.5 g. With intraplacental injection a similar effect was seen, and again there was little effect on the placental weight even though a very much larger dose was administered by this method. In a 22-day pregnant rabbit there were 5 foetuses. One of these was given an intraplacental injection of 1 mg. TME and the others remained as controls, 2 of them receiving injections of normal saline. The mother was killed 5 days after injection and the foetuses and placentae removed for examination. At the injected site the foetus was dead; it weighed 7 g. and the placenta 9 g. All the controls were alive and appeared normal, the average foetal weight being 43 g. and the average placental weight 10 g. There was no apparent difference between the untreated controls and the controls injected with saline.

The intra-amniotic injection of 10 μg. TME killed 3 out of the 5 foetuses injected. As the volume of the intra-amniotic fluid at this stage of pregnancy—around 20 days—was about 5 ml. this dose is roughly equivalent to a subcutaneous dose of 2–4 mg./kg. to the doe. With intraplacental injection the dose required to kill the foetus is about ten times greater, suggesting that the toxic effect is primarily on the foetus and not on the placenta.

Podophyllotoxin was also investigated by this method (at a slightly earlier stage of pregnancy, around 17 days) and the results are shown in Table IV. This drug also appears to exert its toxic effect directly on the foetus and not on the placenta.

The Action of p-*(Di-2-chloroethylamine)-phenyl Butyric Acid on Pregnancy in Mice*

The aromatic nitrogen mustard *p*-(di-2-chloroethylamine)-phenyl butyric acid (CB 1348; chlorambucil) synthesized by Everett, Roberts, and Ross (1953) inhibits the transplanted Walker Rat Tumour 256 (Ross, Davis, Roberts, and Everett, 1952) and is effective against certain malignant lymphomas (Galton, Israels, Nabarro, and Till, 1955). It was tested for its effect on pregnancy, and the results are shown in Table V. The sodium salt of CB 1348 was prepared by dissolving the mustard in N/50 sodium hydroxide in methanol and evaporating *in vacuo* in a tepid water bath. The residual gum was dissolved in saline and injected within an hour of preparation, the total dose being given in a single injection.

TABLE V

THE EFFECT OF *p*-(DI-2-CHLOROETHYLAMINE)-PHENYL BUTYRIC ACID ON PREGNANCY IN MICE

Duration of Pregnancy	Route of Administration	Dose (mg./kg.)	Maternal Mortality	Effects on Foetus
12–13 days	Intraperitoneal	10	0/12	35 live (average weight 1·66 g.) and 18 dead (average weight 1·0 g.) mice delivered on 20th day
	,,	20	0/13	Dead litter at term. Average weight 0·59 g.
	Intravenous	20	0/2	Dead litter at term
	Intraperitoneal*	20	0/2	Dead litter at term. Average weight 0·63 g.
	,,	30	0/2	Dead litter at term. Average weight 0·65 g.
	,,	40	0/3	Dead litter at term
7	,,	20	0/5	Reabsorption of foetuses
1	,,	20	0/12	Pregnancy prevented in all animals
1	—	Nil	0/10	8 out of 10 had normal litters

* Ovariectomized on the 12th day of pregnancy and treated with progesterone 1·5 mg./day.

The characteristic effect of this mustard, when administered on the 12th or 13th day, is to produce a dead litter at term. This occurs with doses of 20 mg./kg. whereas a dose of 10 mg./kg. results in both normal and stillborn offspring.

The LD50 was 45 mg./kg., in an experiment with 90 mature non-pregnant mice, and therefore the therapeutic ratio is low, being of the order of 2. During this experiment it was noticed that, whereas a dose of 40 mg./kg. produced no maternal deaths, a dose of 48 mg./kg. resulted in a 100% mortality.

Two mice were ovariectomized on the 11th day and pregnancy was maintained by the daily administration of 1.5 mg. progesterone. This is ample to maintain gestation in ovariectomized mice (Robson, 1938). The first dose of progesterone was given on the day preceding ovariectomy and the CB 1348 on the day following ovariectomy. Removal of the ovaries did not alter the effect produced by the mustard.

When CB 1348 was administered on the 7th day absorption of pregnancy occurred. Consideration of the weights shows that development ceased soon after injection of the mustard. Laparotomy performed on the 16th day showed complete reabsorption of all foetuses. If a dose of 20 mg./kg. is given before implantation has occurred, i.e., before the sixth day, pregnancy is prevented.

Effect of CB 1348 on the Foetus.—The dead foetuses were all well below the size of normal live offspring and all showed various degrees of arrested development. Not all the foetuses were obtained for examination, as many were eaten by the mothers soon after delivery.

Examination showed cleft palate and non-expansion of the lungs to be the two most constant features. Abdominal hernias, enlargement of the spleen, absence of buccal opening, and the presence of limb buds instead of hind legs were also found.

Of the 30 live mice from the two groups in which the mothers received 10 mg./kg., five were eaten by the mother within the first 10 days of life. The remainder have been observed for three months, animals being killed and examined at various times. With two exceptions, all have developed normally, and have been interbred, with the production of normal litters. The two exceptions were both from the same mother and were very small at birth, 1.04 g. and 0.98 g. The smaller one was placed with a foster-mother, but still failed to develop. It died 25 days after birth, but post-mortem showed no abnormality. The other mouse has grown slowly and only weighed 10 g. when 60 days old as compared with 16 g. for other mice in this group.

As closure of the palate occurs on the 14th–15th day of gestation in the mouse, two animals were injected with CB 1348 20 mg./kg. on the 16th day of pregnancy. Each produced a live and normal litter at term.

Effect of Other Compounds on Pregnancy

Various other compounds were tested on mice 11–13 days pregnant and on rabbits 14–15 days pregnant. The substances used were D-glucosamine hydrochloride (Quastel and Cantero, 1953), 4-amino-N^{10}-methyl pteroyl glutamic acid, 2:4:6-tris-ethylene-imino-1:3:5-triazine (triethylene melamine) (Hendry, Homer, Rose, and Walpole, 1951), Actinomycin C, 6-mercaptopurine and four dimethane sulphonyloxyalkanes $CH_3SO_2O(CH_2)_nOSO_2CH_3$ (Haddow and Timmis, 1953). Those tested were the compounds where $n=3$, 4, 6, and 8. The compound where $n=4$ is known as Myleran. None was more than slightly active at this stage of pregnancy, and then only at a dose level which caused maternal deaths, except triethylene melamine and 6-mercaptopurine. Triethylene melamine interrupted pregnancy in 3/8 mice treated with 0.8 mg./kg. of this compound, with no maternal deaths; a dose of 1.5 mg./kg. interrupted pregnancy in 6/8 mice, but killed 2 of the mothers. 6-Mercaptopurine interrupted pregnancy in 6/6 mice treated with doses ranging from 150–250 mg./kg. There were no maternal deaths with these doses, but a dose of 300 mg./kg. killed some of the mothers.

6-Mercaptopurine and Actinomycin C were also given by intra-amniotic and intraplacental injection. The former compound only produced an effect with doses of 1 mg. and then not all the foetuses were affected. Actinomycin interrupted pregnancy at 5/5 sites with a dose of 10 μg. injected into the amniotic fluid, smaller amounts only occasionally producing the effect. Doses up to 700 μg. into the placenta had no effect on pregnancy.

Discussion

Nucleotoxic substances, given orally or by injection, differ considerably in their ability to interrupt pregnancy, and this difference bears some relation to their mode of action. By far the most active are the spindle poisons podophyllotoxin and the colchicine derivatives, all these substances being consistently capable of interrupting pregnancy in doses which are not toxic to the mother. The therapeutic ratio, however, varies considerably with the different substances.

The chromosomal poisons *p*-(di-2-chloroethylamine)-phenyl butyric acid (CB 1348), tris-ethylene-imino-1:3:5-triazine, and the dimethane sulphonyloxyalkanes are much less active and, with the exception of CB 1348, only produce an effect with doses which are also lethal to the mother.

The antimetabolites 6-mercaptopurine, D-glucosamine and 4-amino-N^{10}-methyl glutamic acid were almost completely inactive when the drugs were given to mice between the 11th and 13th days of pregnancy. This is supported by the work of Thiersch (1954) with 6-mercaptopurine; he found that when the drug was given to rats on the 12th–13th days there was little effect on the pregnancy although stunted foetuses were produced. However, the foetus was much more sensitive at the time of implantation—7th–8th day—when 90% foetal deaths were produced.

Similarly aminopterin, a folic acid antagonist, has been shown by Thiersch and Philips (1950) to

interrupt pregnancy in rats and mice in doses which are not lethal to the mother. The embryos were much more sensitive to the drug before the 10th day of pregnancy. Thiersch (1952) has also demonstrated a similar effect in the human subject. Abortion can be produced during the first 3 months of pregnancy with doses which have only a mild transitory effect on the haemoglobin and white cell count of the mother.

With the spindle poisons the therapeutic ratio in the pregnancy tests—ratio of dose which will consistently interrupt pregnancy to dose which kills some of the mothers—bears some relation to the ratio of the maximum tolerated dose to the minimum effective dose when the drugs are used in the treatment of neoplasms. The values of the former ratio with colchicine, TME, and demecolcin are respectively 1, 12, and 4, and for the latter ratio 2, 50, and 10. There is no such relationship with the other substances tested.

Our results indicate that a more satisfactory test for the cytotoxic effect of these substances is that of injection directly into the amniotic fluid. The effect is produced by a direct action on the foetus and not by an indirect effect through the placenta, as both intra-amniotic and intraplacental injections have very little effect on placental weight. Furthermore, to interrupt pregnancy by intraplacental injection a very much larger dose is needed than that which is effective by the intra-amniotic route.

As has already been seen the intra-amniotic injection of TME produces results which are comparable to those obtained by systemic injection, and the same applies to podophyllotoxin. Actinomycin C and 6-mercaptopurine are also effective by this method, whereas parenteral administration of the former produces toxic effects on the mother before producing a demonstrable effect on pregnancy.

All these experiments, therefore, suggest that the therapeutic ratio is essentially of value only in the testing of drugs which are spindle poisons, whereas the intra-amniotic injection method may also provide information about other cytotoxic drugs.

Summary

1. The action of various nucleotoxic drugs on pregnancy in mice and rabbits has been investigated.

2. The ability of these drugs to interrupt pregnancy bears a definite relation to their mode of action and to the stage of pregnancy at which they are administered.

3. By far the most effective are the spindle poisons, podophyllotoxin and the colchicine derivatives. With these drugs there is a definite relation between the therapeutic ratio as determined by the pregnancy test and that found by testing the anti-tumour activity of these compounds.

4. Intra-amniotic and intraplacental injection of various nucleotoxic drugs was performed to determine the site of action of these nucleotoxic substances. They probably act directly on the foetus and not via the placenta.

5. Intra-amniotic injection seems to be a simple and reliable method of testing a drug for cytotoxic properties.

We are grateful to Professor F. Bergel for the supply of CB 1348; Dr. C. W. French for TME; Professor R. Meier for demecolcin; Dr. F. Prescott for 6-mercaptopurine; Dr. F. L. Rose for triethylene melamine; Dr. H. A. Wilpert for Actinomycin C; Dr. F. H. Brain for advice on the preparation of solutions of CB 1348, and the Medical Research Council for a grant (to J. M. R.) which defrayed in part the expenses of this investigation.

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THE EFFECT OF MUSCARINE ON PERFUSED SUPERIOR CERVICAL GANGLIA OF CATS

BY

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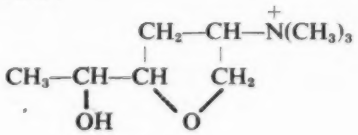
In 1914 Dale described the actions of acetylcholine as being of two distinct types, namely, muscarinic and nicotinic. He defined the muscarinic action as "the action which true muscarine exhibits in its pure form, uncomplicated by the nicotine action." The muscarine effects of choline esters were "purely peripheral in their origin, unaffected by nicotine in large doses, but readily abolished by small doses of atropine." This useful classification has been widely used ever since. With increasing knowledge of the physiology of the autonomic nervous system, it is now possible to define nicotinic effects as the effects of acetylcholine and other drugs at ganglionic synapses of both sympathetic and parasympathetic ganglia, and also at neuromuscular junctions; and muscarinic actions as actions on the effector cells innervated by postganglionic cholinergic fibres, mostly in parasympathetic nerves.

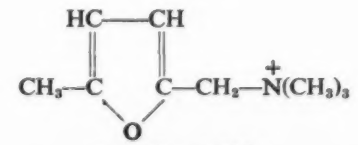
Reasons have been given previously (Ambache, 1949) for suspecting that natural muscarine may have actions other than those hitherto strictly defined as "muscarinic." During an investigation of the pharmacology of the synthetic compound 2268F (acetal of 2:3-dihydroxypropyl trimethylammonium iodide), thought to be isomeric with muscarine,* it was found that, although endowed with intense muscarinic activity, this compound also exhibited nicotinic effects on striated muscle and sympathetic ganglia. The ganglionic effect was shown to be antagonized by atropine, and the suggestion was made that in experiments on the blood pressure of atropinized cats this type of antagonism might well prevent the detection of the nicotinic effects of 2268F and of similar compounds (as in the experiments of Fourneau, Bovet, Bovet, and Montezin, 1944) and possibly also of muscarine itself. An analogous situation was described later by Ing, Kordik, and Tudor Williams (1952) for furmethide (furfuryl trimethylammonium iodide) and for its 5-methyl derivative. Both these compounds have powerful muscarinic actions; they also display some degree of nicotinic activity on the perfused superior cervical ganglion. Yet, even in large doses, neither produces a rise in blood pressure in the atropinized cat. Root's (1951) experiments with pilocarpine also suggest that a pressor action of this substance may have been masked by atropine. Furthermore, the ganglion-blocking action of atropine may antagonize even the effect of acetylcholine, as found in denervated ganglia by Konzett and Rothlin (1949). Several examples of this type of ganglionic action of atropine have been tabulated in a previous paper (Ambache, 1954). We are thus faced, firstly, with a considerable body of evidence suggesting that atropine may block not only muscarinic effects but also certain nicotinic effects of muscarine-like substances, and, secondly, with the possibility that muscarine itself may have certain nicotine-like actions.

We have therefore examined the effect of a sample of chromatographically purified, crystalline, muscarine chloride on perfused preparations of the cat's superior cervical ganglion. A preliminary account of these results has appeared elsewhere (Ambache, Perry, and Robertson, 1953). Later, Waser (1955)

* According to Eugster (1956) the empirical formula for muscarine is $C_9H_{20}O_2N$ and not, as thought by Kögl, Duisberg, and Erxleben (1931), $C_8H_{18}O_2N$. Eugster's careful work suggests that muscarine is a salt of trimethyl-[2-(α-hydroxyethyl)-tetrahydro-furyl-(4)]-ammonium. The resemblance to 5-methyl-furmethide is shown in the formulae below:



Muscarine



5-me-furmethide

found that another sample of muscarine chloride prepared by Eugster and Waser (1954) had no pressor, "nicotinic," effect on the cat's blood pressure, but his experiments were conducted in the presence of atropine. When, however, Konzett and Waser (1956) examined the activity of Eugster and Waser's sample of muscarine on perfused preparations of cats' cervical ganglia, they obtained results which were identical with ours, and also showed that a subthreshold dose of muscarine may potentiate responses to acetylcholine and to preganglionic stimulation.

METHODS

Muscarine.—A sample of highly purified crystalline muscarine chloride, prepared from *Amanita muscaria* at the Wellcome Research Laboratories, was sent to us through the courtesy of Dr. S. Wilkinson and Dr. J. W. Trevan. When tested on guinea-pig ileum preparations this sample was active at a threshold concentration of 3×10^{-9} (Ambache and Lessin, 1953; 1955).

Perfusion of the Superior Cervical Ganglion.—Cats were anaesthetized with 40 mg./kg. pentobarbitone sodium intraperitoneally or, in a few experiments, with ether followed by intravenous chloralose (80 mg./kg.). The right superior cervical ganglion was prepared by the method described by Kibjakow (1933), with modifications suggested by Feldberg and Gaddum (1934), and was perfused with aerated Locke solution containing 2 g./l. of glucose and filtered through sintered glass (No. 4 Jena). The Locke solution was warmed by passage through a plastic tube lodged in the oesophagus, as suggested by MacIntosh (personal communication). Otherwise apparatus and method were the same as used by Ambache (1949), except that the linen filter in the perfusion circuit was replaced by a small sintered glass filter. Blood pressure was recorded from a femoral artery. Intravenous injections were made into the contralateral femoral vein.

Denervated Ganglia.—In an initial aseptic operation under pentobarbitone sodium anaesthesia the right, or sometimes both, superior cervical ganglia were decentralized by avulsion of about 1 in. of the vagosympathetic trunk low in the neck. The denervated ganglia were perfused after an interval varying from 11 days to 5 months.

RESULTS

Action of Muscarine on Normal Ganglia.—As is well known, very small doses of muscarine, given intravenously, cause a large fall in blood pressure in animals which have received no atropine. Muscarine was therefore administered to the ganglion by injection into the perfusion fluid. Injection of muscarine in this way into normal ganglia was followed by a contraction of the nictitating membrane in 14 out of 16 experiments. The effective dose of muscarine varied considerably. Thus in the fourteen experiments in which muscarine produced a contraction the doses were: in eight, 20–187 μg.; in three, 1.6–4 μg.; and in another three, 0.1 μg. An experiment on one of the most sensitive preparations is illustrated in Fig. 1. In this experiment the femoral blood pres-

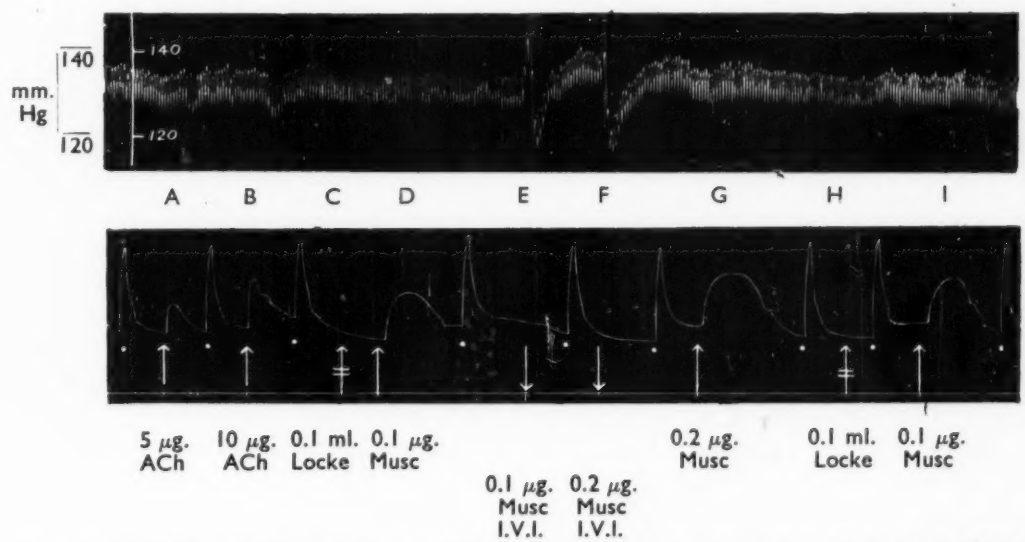


FIG. 1.—Cat, perfusion of innervated superior cervical ganglion. Effect of pure muscarine. Above, femoral blood pressure; below, contractions, upwards, of the nictitating membrane. At the dots, maximal preganglionic stimulation applied for 5 sec. Muscarine: 0.1 μg. at D and I, and 0.2 μg. at G, administered to the perfused ganglion; and 0.1 μg. intravenously at E and 0.2 μg. at F (downward arrows). Acetylcholine at A and B, and Locke solution at C and H, into the perfusion stream.

sure was also recorded. Muscarine in a dose of 0.1 μg. produced a contraction of the nictitating membrane, which was roughly equal in size to that produced by the injection of 10 μg. of acetylcholine, but which developed more slowly, after a longer latency (9 sec.), and which was of longer duration. In this experiment the ganglion was rather less sensitive at the start of perfusion, requiring doses of 0.5–1 μg. of muscarine for contractions to be elicited.

This increasing sensitivity during prolonged perfusion was noticed in several other experiments. In one cat 20 μg. of muscarine was quite without effect at an early stage of the perfusion, although later on a threshold response was obtained with 0.2 μg. This gradual sensitization of the ganglion may be due to a cumulative effect of muscarine. In the 2 experiments in which we failed to get any response to muscarine the drug was given only at the start of the perfusion and the dose was not increased above 20 μg. owing to the scarcity of material. From Fig. 1 it is apparent that the potency of muscarine relative to acetylcholine was of the order of 100:1, but values of this ratio varying from 200:1 to 1:20 were observed. The varying ratio could, however, almost wholly be accounted for by variations in the sensitivity to muscarine. Thus the ratio not only varied from cat to cat but also tended to increase as the perfusion proceeded in any one cat.

Control injections of Locke solution did not produce stimulation in any of the experiments.

Action of Muscarine on Denervated Ganglia.—Muscarine stimulated all of 13 ganglia which had been decentralized for periods long enough to allow degeneration of their preganglionic nerve supply. This showed that the ganglionic effect of muscarine could not be attributed to indirect stimulation of preganglionic nerve fibres and endings. As a group, the denervated ganglia appeared to be more consistent in responding to low doses of muscarine than the normal ganglia, of which only a small proportion had responded to as little as 0.1 μg. muscarine. In fact *all* the denervated ganglia responded to small doses of muscarine—five to 0.2–0.5 μg.; seven to 2–4 μg.; and one to 12.5 μg. Thus in the denervated ganglia the threshold dose for stimulation was rarely greater than 3 μg. It appears, therefore, that the occasional relatively insensitive normal ganglion may be rendered sensitive by denervation. We did not observe much increase in the sensitivity to acetylcholine after denervation, and, on the average, the dose required for stimulation was some one-third to one-half of that required in normal ganglia, there being a considerable overlap in the effective doses in the two groups.

Comparison with Choline.—The most common pharmacologically active impurity in extracts of *Amanita muscaria* is choline; in fact such extracts may contain 20 times as much choline as muscarine (King, 1922). Although our specimen of muscarine was believed to be pure, we tested the effects of choline injected in the same way as the muscarine (Fig. 2). It will be seen that 75 μg. choline was required to produce a contraction equivalent in size to that produced by 0.1 μg. muscarine. Thus, the effect of the crystalline material could not be due to traces of choline in it, since, even if it had consisted entirely of choline and had contained no muscarine at all, it would then have had no effect. In other experiments muscarine was 200–500 times as active as choline.

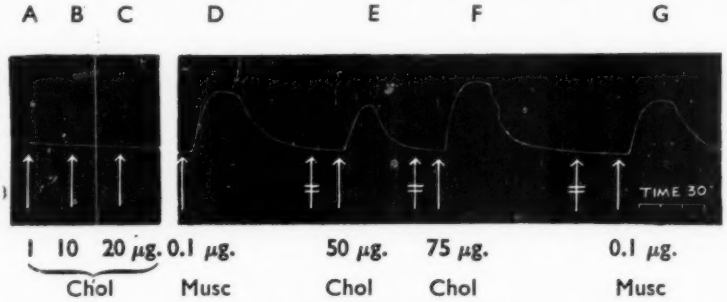


FIG. 2.—Cat superior cervical ganglion, preganglionically denervated 40 days previously. Comparison of muscarine with choline. All injections into the perfusion stream. Choline, μg.: at A, 1; B, 10; C, 20; E, 50; and F, 75. Muscarine, 0.1 μg. at D and G. Control injections of 0.15 ml. Locke solution (barred arrow) before E, F, and G.

Site of Action of Muscarine.—The contractions of the nictitating membrane produced by the injection of small doses of muscarine into the perfusion stream of the isolated superior cervical ganglion were most likely to have been due to a stimulant action of muscarine on the ganglion cells. Nevertheless, the following additional evidence was obtained to show that its site of action was truly ganglionic.

It is known that the cat's nictitating membrane receives both adrenergic and cholinergic fibres from the postganglionic cervical sympathetic nerve (Bacq and Fredericq, 1935); and, indeed, the smooth muscle in the membrane responds to acetylcholine, an effect which is classifiable as muscarinic. If, therefore, muscarine were to escape out of the perfused ganglion and to reach the nictitating membrane itself, it might produce a

local contraction unrelated to any ganglion-stimulant effect. We have attempted to exclude this possibility in several ways. We have noticed in a number of experiments that it seems to be virtually impossible to achieve complete isolation of the perfused tissue from the general circulation. In about 10% of cats this is due to patency of the internal carotid artery, a condition described by Davis and Story (1943). The existence of a patent internal carotid artery can be detected by momentarily lowering the perfusion pressure to zero and observing whether there is a reflux of arterial blood into the perfusion cannula. When reflux occurred the internal carotid artery was looked for and tied. Even in the absence of a patent internal carotid, and of reflux, the ganglionic perfusate is always slightly tinged with blood when the perfusion pressure is below a certain critical level, indicating that there must be small vascular connexions between the ganglion and the general circulation. These, almost certainly, are situated in the postganglionic trunk, which has to be left intact. On raising the perfusion pressure above the critical level the venous effluent becomes clear, but the possibility then exists of some perfusate entering the general circulation. Some idea of the extent of this leakage into the general circulation may be gained by taking concurrent records of the blood pressure. We did, in a few experiments, observe a fall in blood pressure after injections of muscarine or of acetylcholine into the perfusion fluid ; but in 5 of the experiments this was completely absent, and in general it was negligible. Thus, Fig. 1 illustrates that the administration to the ganglion of 0.2 μg. muscarine, which elicited a large contraction of the nictitating membrane, was almost without effect on the blood pressure, whereas 0.1 μg. muscarine, when injected intravenously, produced a considerable depressor effect and very little action on the nictitating membrane.

Further evidence for the ganglionic site of action of the muscarine in these experiments was obtained from the following study of its interaction with other drugs.

Antagonism of the Ganglionic Action of Muscarine by Atropine Administered to the Ganglia.— Atropine in very small doses blocked the stimulant action of muscarine on the ganglion. This effect was reversible and was obtained both in normal and in denervated ganglia, as illustrated in Figs. 3 and 4. In the experiment of Fig. 4, on

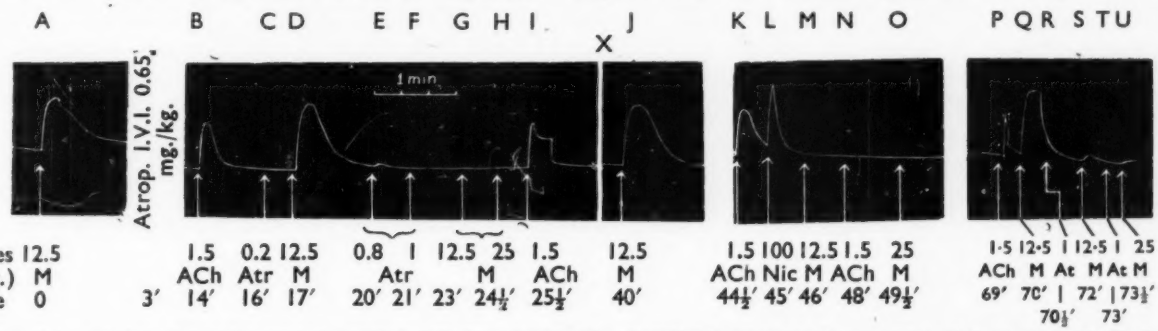


FIG. 3.—Cat, perfusion of superior cervical ganglion, 142 days denervated. Ganglionic responses to muscarine 12.5 μg. at A, D, G, J, M, Q, and S, and 25 μg. at H, O, and U; to acetylcholine 1.5 μg. at B, I, K, N, and P, all injected into the perfusion system. Atropine 0.65 mg./kg. administered intravenously to the cat 11 min. before B. Atropine administered to the perfused ganglion: 0.2 μg. at C, 0.8 μg. at E, and 1 μg. at F, R, and T. At L, 100 μg. nicotine to perfusion. Drum stopped at X. Timing of doses given below in min.

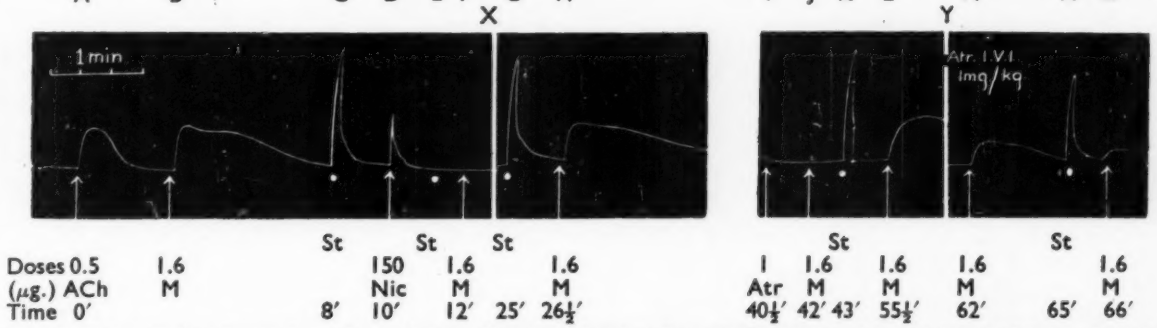

A B C D E F G H I J K L M N O
X
Y
1 min
Atr. I.V.I. 1mg/kg
St St St St St
Doses 0.5 1.6 150 1.6 1.6 1 1.6 1.6 1.6 1.6
(μg.) ACh M Nic M M Atr M M M M
Time 0′ 8′ 10′ 12′ 25′ 26½′ 40½′ 42′ 43′ 55½′ 62′ 65′ 66′

FIG. 4.—Cat, perfusion of normal superior cervical ganglion. Injections into the perfusion stream: at A, acetylcholine 0.5 μg.; muscarine 1.6 μg. at B, F, H, J, L, M, and O. At the white dots maximal preganglionic stimulation for 5 sec. At D, 150 μg. nicotine, and at I, 1 μg. atropine, administered to the ganglion. Atropine 1 mg./kg. intravenously before M (at Y). Drum stopped at X and Y; timing of doses given below in min.

a normal ganglion, after a dose of 1 μg. atropine was administered into the perfusion fluid at I, the response to a previously effective dose of muscarine (1.6 μg.) was completely abolished (J). This dose of atropine had no effect on the response to preganglionic stimulation (K), and its blocking effect to muscarine itself had completely passed off in 15 min. (L). A dose of 1 mg./kg. of atropine given intravenously (before M) produced, in this experiment, some depression of the response to muscarine and to preganglionic stimulation. This was attributed to some reflux, in this experiment, of atropine from the general circulation into the perfused ganglion, since in several other experiments similar doses of atropine given intravenously had no such depressant action on the ganglionic response to muscarine, as can be seen in the experiment of Fig. 3. These observations again excluded an action of muscarine on the nictitating membrane since the ganglionic effect of muscarine was obtained when the rest of the cat was atropinized, including its nictitating membranes.

Small doses of atropine did not block the effects of injected acetylcholine to the same extent as they blocked the effect of muscarine (Fig. 3); nevertheless, slightly larger doses (1–10 μg.) did block injected acetylcholine and still larger doses (100 μg.) could block the effects of preganglionic stimulation as well.

Suppression of Muscarine Responses by Ganglion-blocking Drugs

Normal Ganglia.—Fig. 4, D–F, shows that when transmission in the ganglion had been completely blocked by 50–100 μg. nicotine, the stimulant effect of muscarine was also totally abolished, but recovered later when normal transmission returned. Similar effects were produced by tetramethylammonium (TMA) when this was substituted for nicotine in other experiments.

On the innervated ganglia 0.1–1 mg. hexamethonium blocked preganglionic stimulation, acetylcholine and muscarine.

Denervated Ganglia.—Preganglionic denervation produced an interesting change in the susceptibility of ganglia to certain blocking agents. Drugs which block by depolarizing ganglion cells (Paton and Perry, 1953), such as nicotine and TMA, never failed to block the ganglionic actions of muscarine or of acetylcholine. On the other hand, hexamethonium and tetraethylammonium (TEA), which are non-depolarizing competitive blocking agents on normal ganglia, were no longer effective in blocking either acetylcholine or muscarine. Whereas in normal ganglia 0.1–1 mg. hexamethonium produced total block, in denervated ganglia doses of 10 mg. were completely ineffective. On the other hand, in denervated ganglia, relatively small doses (0.5–1 mg.) of hexamethonium and TEA still blocked the stimulant effects of nicotine, of the 3-bromophenyl ether of choline, and of TMA completely. Some of these findings are shown in Fig. 5, which illustrates the block produced by successive doses of hexamethonium to TMA and nicotine but not to acetylcholine or muscarine. These findings were studied more extensively by Perry and Reinert (1954).

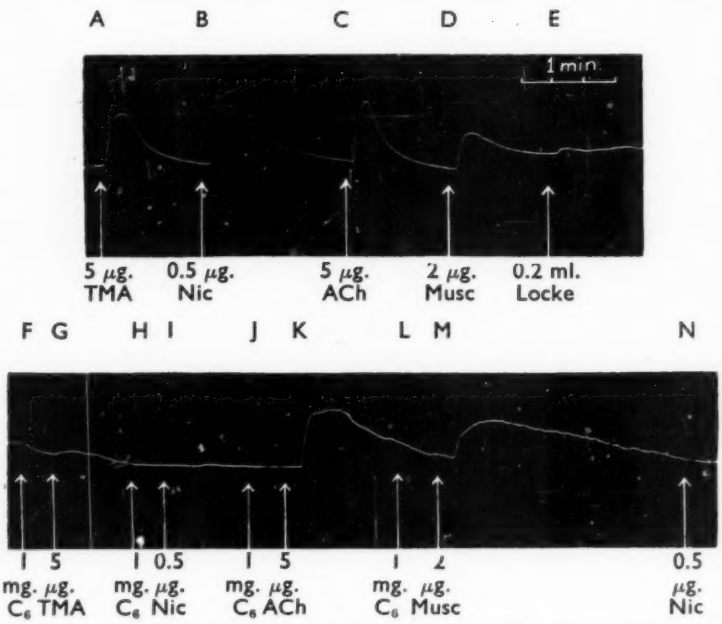


FIG. 5.—Cat, perfusion of superior cervical ganglion 15 days after preganglionic section. Stimulating effects of 5 μg. tetramethylammonium at A, 0.5 μg. nicotine at B, 5 μg. ACh at C, and 2 μg. muscarine at D. These 4 doses are repeated at G, I, K, and M, each one preceded at an interval of 1 min. by 1 mg. hexamethonium. Hexamethonium blocks nicotine and TMA, but not ACh or muscarine. Nicotine was repeated at N, 9 min. after the last dose of hexamethonium, and was still blocked. Control injection of 0.2 ml. Locke solution at E.

DISCUSSION

Muscarine, like acetylcholine, is capable of stimulating the ganglion cells of the cat's superior cervical ganglion. The fact that muscarine itself has such an action suggests to us that it might be preferable, in future, to use the term "parasympathomimetic" when referring to actions hitherto known as "muscarinic."

The ganglionic stimulant action of muscarine is blocked by atropine even in very small doses. Likewise, atropine has some effect, even in normal ganglia, in depressing the response to acetylcholine. The doses of atropine required are not large and the action must be regarded as a specific one. Moreover, the ganglion-stimulant effects of muscarine are also blocked, in normal ganglia, by all the usual ganglion-blocking agents, both depolarizing and competitive.

The fact that, in both normal and denervated ganglia, muscarine stimulation is abolished by nicotine is strong supporting evidence that the effect of the drug is truly ganglionic. This is also borne out by the results with other ganglion-blocking drugs. However, after denervation a remarkable change was observed, for which no simple explanation is at present forthcoming.

Hexamethonium, which before denervation completely blocked the actions of all the ganglion-stimulating drugs, failed after denervation to block acetylcholine and muscarine, although still fully effective against nicotine and TMA. This finding seems to argue that the changes in the cell membrane after denervation are such that the receptors for acetylcholine and muscarine are no longer affected by hexamethonium, while those for nicotine and TMA remain susceptible to this drug. If this is the correct interpretation, it implies that the membrane of the denervated ganglion cell is differentiated and contains at least two types of receptor. Paton and Perry (1953) have discussed the possibility that the ganglion cell membrane is differentiated into specifically reactive patches of membrane which are the site of a local depolarization, and adjacent parts of the membrane excited by electrotonic spread. It is conceivable that a similar differentiation may explain the present drug effects.

Župančič has propounded the hypothesis (see references in Župančič and Majcen, 1956) that the receptors for acetylcholine are very similar to, if not identical with, cholinesterase (ChE). According to this hypothesis, the receptor protein at sites where acetylcholine exerts a nicotinic action would resemble aceto-ChE, as both are known to be depressed by an excess of substrate. At "muscarinic" sites the receptor would resemble butyro-ChE, as neither is inhibited by substrate excess. According to Župančič some of the changes in the pharmacology of skeletal muscle after denervation are explicable by assuming an alteration in the receptor protein towards the butyro-ChE type, and it is conceivable that a similar change may occur in denervated ganglia. In histochemical studies on various ganglia in cats, including the superior cervical, Koelle (1950, 1951) reported that, after preganglionic denervation, aceto-ChE had almost completely disappeared, except in occasional ganglion cells. On the other hand, the butyro-ChE remained abundantly visible throughout the histological sections. Were the muscarinic site of action in denervated ganglion cells predominantly of the butyro-ChE type, hexamethonium would not block muscarine or acetylcholine, since it does not block the action of these drugs at the "muscarinic receptors" in smooth muscle, which are also of the butyro-ChE type. Moreover, Župančič has shown that atropine can block the butyro-ChE but not the aceto-ChE; this would also fit with the results we obtained on the ganglia. Excess of nicotine, on the other hand, appears to render the ganglion cells inexcitable to all drugs, whatever their receptor sites.

Muscarine was shown by Dale and Gasser (1926) to be devoid of nicotinic action at the neuromuscular junction. We have in a few unpublished experiments confirmed this, using the frog's rectus abdominis, the leech dorsal muscle, and the pigeon's iris; Dr. E. Zaimis (personal communication) has also confirmed it in an experiment on a normal cat's tibialis anticus. Thus muscarine appears to belong to a group of drugs, all of which possess an action at ganglionic synapses but not at normal neuromuscular junctions. The other members of this group are arecoline (Dale and Gasser, 1926; Feldberg and Vartiainen, 1934), pilocarpine and acetyl-β-methyl choline (unpublished observations).

Summary

1. The action of a sample of highly purified muscarine chloride, injected into perfused superior cervical ganglia of cats, has been studied.

2. Precautions were taken to reduce to a minimum the leakage of muscarine into the general circulation after injection into the ganglion perfusion. Blood pressure records showed that such leakage was absent in 5 experiments in which ganglia were stimulated by muscarine. In some of these the effective intraganglionic dose of muscarine was one which, when given intravenously, was too small to affect the nictitating membrane directly.

3. Stimulation by muscarine occurred in ganglia of which the preganglionic nerve supply had been cut and allowed to degenerate. A higher proportion of such ganglia than of normal ganglia responded to low doses of muscarine.

4. Ganglionic stimulation by muscarine persisted after atropine was administered to the cats systemically, but it was abolished reversibly by 1 μg. or less of atropine administered to the ganglion.

5. Muscarine was 200–500 times as active as choline, thus excluding the possibility that the muscarine effect could have been due to contaminant traces of choline.

6. In normal and denervated ganglia stimulation by muscarine was always reversibly abolished by ganglionic infusion of 50–100 μg. nicotine.

7. In normal ganglia 0.1–1 mg. hexamethonium blocked the stimulating effect of muscarine, acetylcholine, nicotine, and other drugs.

8. In denervated ganglia 1–10 mg. hexamethonium failed to block muscarine or acetylcholine, though it blocked equiactive doses of nicotine, tetramethylammonium, and the 3-bromophenyl ether of choline.

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FORMATION OF CATECHOL AMINES DURING SPLANCHNIC STIMULATION OF THE ADRENAL GLAND OF THE CAT

BY

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In 1951 Hökfelt found that, following depletion of the catechol stores of the adrenal by insulin, several days were required for complete restoration of the normal amine content. He concluded from his observations that the rate of synthesis of adrenaline must be slow. Recently Udenfriend, Cooper, Clark, and Baer (1953) and Udenfriend and Wyngaarden (1956) observed a very low rate of incorporation of ^{14}C into adrenaline and noradrenaline after the injection of ^{14}C-labelled tyrosine or phenylalanine. However, after administration of ^{14}C-labelled dopa, the incorporation was rapid. The calculated half-lives of both amines were of the order of 3–6 days. This low turnover suggested that at least one step in their formation was slow. Since the turnover rates of noradrenaline and adrenaline were equal, the methylation of noradrenaline could not be a limiting factor.

In the foregoing papers no observations were made on the effect of electrical stimulation of the splanchnic nerves. Elliott (1912) described an extensive investigation of this, though he assumed that only one active amine was concerned. In his first experiments he divided the splanchnic nerves on one side and observed the effect of administering morphine, ether, chloroform and other drugs. He found that the innervated gland which still received impulses from the brain was always depleted of amines as compared with the gland the nerve fibres to which had been cut. In contrast to these experiments there were others in which he divided the splanchnic nerves on both sides, and then applied electrical stimulation for periods up to 7 hours to those of one side. When he removed the glands at the end of this time he found little or no depletion of the amine store of the stimulated side. He said: "So slight is the change in the residual adrenalin caused by faradization of the splanchnic nerves that it would never have sufficed to convince me of the existence of the splanchnic control." Elliott's results suggested that in these experiments there must have been a vigorous formation of noradrenaline and perhaps also of adrenaline during the period of stimulation, for he recorded the blood pressure throughout and observed that it was continuously raised.

In his paper Elliott quoted Tscheboksaroff (1910), who also stimulated the splanchnic nerves and "in a very clear series of experiments . . . observed that, after stimulation, the gland on the stimulated side yielded a more potent extract, that is contained more adrenalin, than that of the other side."

In order to obtain more information on the rate of formation of noradrenaline and adrenaline experiments have been carried out in which the effects of splanchnic stimulation on medullary outflow and amine content of the cat's adrenal were determined. Results have been obtained which are interpreted to mean that splanchnic stimulation increases both the rate of synthesis and the methylation of noradrenaline.

METHODS

Cats under ether were given 80 mg./kg. chloralose intravenously. The stomach, intestines, pancreas, and spleen were removed, and the blood supply to the liver through the hepatic artery and portal vein was arrested. By dissection upwards under the diaphragm, the left splanchnic nerves were exposed and cut; the peripheral ends were prepared for stimulation with shielded platinum electrodes. The right splanchnic nerves were similarly exposed and cut. Stimulation was applied for 5 min. from an induction coil, the primary circuit being interrupted by Lewis's rotary contact breaker at a rate of 20/sec. Both vagi were cut, and the blood pressure was recorded from the carotid artery. The outflow of medullary amines was estimated by matching as closely as possible the blood-pressure response to splanchnic stimulation by infusing a mixture of equal parts of adrenaline and noradrenaline at a constant rate. The mixture was infused into the external jugular vein. The comparison of the effect of stimulation with that of infusion was made both by the height of the rise of blood pressure and also by the area of the rise deter-

mined with a planimeter. The two methods gave approximately the same results.

After 10 periods of stimulation for 5 min. at 5-min. intervals, both glands were removed as rapidly as possible, and ground with sand in 0.025M-HCl. The *p*H was adjusted to 5.5 by the addition of sodium acetate. The suspensions were placed in a boiling water bath for 3 min. and they were filtered and the filtrates kept for assay. The quantity of amines as well as the proportion of adrenaline and noradrenaline in the preparations were determined by the method of Burn, Hutcheon, and Parker (1950), using the blood pressure and nictitating membrane of the spinal cat. In a number of experiments the total amine content was determined by the chemical method of Euler and Hamberg (1949).

For the calculation of the results obtained a modification of the method of Gaddum and Lembeck (1949) was employed. The total vasoconstrictor amine content—T.A.—was calculated from the relation

$$\text{T.A.} = \frac{\text{Ae}}{\alpha \times \text{Nf} + \text{Af}} \times 100$$

where Ae is the total activity on the blood pressure expressed as μg. adrenaline; Nf and Af are the percentages of noradrenaline and of adrenaline in the sample as determined from the responses of the nictitating membrane and of the blood pressure, and α is the ratio of noradrenaline activity to adrenaline activity on the blood pressure. Values obtained were expressed as μg. amine/g. tissue wet weight. The right unstimulated gland was assumed to represent a valid control in these experiments. Evidence in favour of the assumption was given by Elliott (1912) and also by Elmes and Jefferson (1942).

Results

Fig. 1 illustrates a typical experiment, as well as the general procedure employed in these studies. At A the left splanchnic nerve was stimulated at a rate of 20/sec. for 5 min. At these high frequencies a progressive deterioration of the medullary discharge was observed. Five minutes after cessation of excitation 25 μg. of a 50–50 mixture of adrenaline and noradrenaline was infused during a 5-min. period (B). At C the stimulation was repeated. This procedure was continued until 10 stimulations and 10 infusions were recorded. In a number of experiments 10 successive stimulations were recorded, with infusions at the beginning and at the end of the experiment. In the particular experiment illustrated, the quantity of amines released from the left gland by splanchnic stimulation amounted to approximately 25 μg./5 min. or 2 μg./kg. cat/min. The figures for all experiments are given in Table I.

Fig. 2 shows a typical experiment illustrating the method for determining both the total amine content and also the proportion of adrenaline in the sample. The details of one experiment were as follows.

Expt. 9 (Table I). Right adrenal 190 mg.; left adrenal 223 mg. The total amine content of the right unstimulated gland was 871 μg./g. by the biological method, and 758 μg./g. by the colorimetric method. The figure for the stimulated left gland was lower, being 515 μg./g. biologically and 466 μg. colorimetrically. Thus, taking the biological figures the stimulated gland had lost 356 μg./g. (Table II). Now the total amine discharged into the blood was estimated as 160 μg., or 713 μg./g. weight of left adrenal gland. Thus, reckoning per g. of left adrenal gland, 357 μg./g. of total amine was newly synthesized, this being

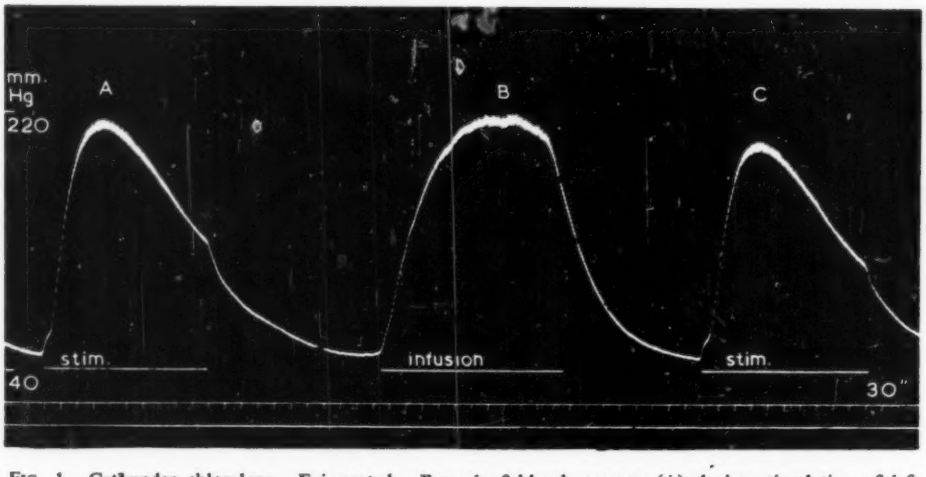

FIG. 1.—Cat under chloralose. Eviscerated. Record of blood pressure (A) during stimulation of left splanchnic nerve for 5 min., (B) during infusion of a mixture of 12.5 μg. noradrenaline and 12.5 μg. adrenaline, (C) during stimulation as before.

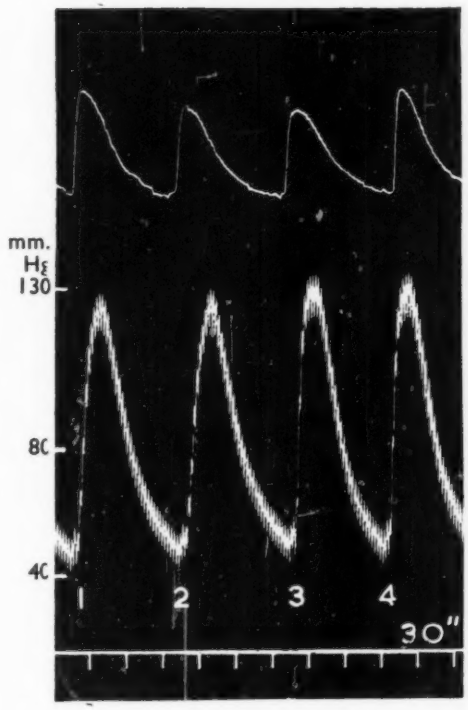

FIG. 2.—Estimation in spinal cat of proportions of noradrenaline and adrenaline in adrenal gland. Upper record, contractions of nictitating membrane; lower record, blood pressure. At 1, 20 μg. adrenaline; 2, 1.7 ml. adrenal extract; 3, 15 μg. adrenaline +5 μg. noradrenaline; 4, 20 μg. adrenaline. The effect of the extract (at 2) on the nictitating membrane was less than that of an equipressor dose of adrenaline (at 1 and 4); hence the extract was less than 100% adrenaline. Similarly the effect of the extract indicated that it contained slightly more than 75% adrenaline injected at 3. Intrapolation showed that it contained 82% adrenaline.

the difference between the amount appearing in the blood and the amount lost from the gland. If the figure for new synthesis is expressed as a percentage of what was present in the unstimulated gland, it is 41% by the biological test and 55% by the colorimetric test.

Table I is a summary of the results obtained with 12 cats, using the procedure just described. Following 10 stimulation periods (total of 50 min.), the mean amine content of the left stimulated gland fell approximately to 75% of that in the right gland. In two experiments (Nos. 6 and 11) there was no appreciable fall, and in one experiment (No. 4) the amine content rose. The results for the percentage of adrenaline in the stimulated gland were of interest because in every experiment this percentage was greater than that in the unstimulated gland. The mean figure for the unstimulated glands was 63%, while that for the percentage of adrenaline in the stimulated glands was 82%.

Formation of Amines.—The amount of pressor amines liberated in the blood by stimulation of the splanchnics is shown in the third column of Table II. The second column gives the loss of amine from the gland, and in every experiment the estimated output was greater than the loss. The excess of the output over the loss is given in the fourth column as the amount of amines synthesized during the experiment. In the final column the amount synthesized is expressed as a percentage of the amine content of the unstimulated gland. The mean figure is 38%.

DISCUSSION

The experiments were designed to obtain an estimate of the amounts of noradrenaline and adrenaline synthesized during splanchnic stimulation. In 12 experiments in which the left adrenal

TABLE I

CHANGES IN AMINE OUTPUT OF STIMULATED ADRENAL GLANDS

Cat	Total Amine Content (μg./g.)		Amine Content of L. Gland as Percentage of R.	Percentage of Adrenaline	
	R.	L.		R.	L.
1	684	284	41·5	50	80
2	638	493	77	75	100
3	1,520	1,320	87	63	75
4	1,080	1,320	122	50	70
5	1,400	1,230	88	50	66
6	438	415	95	70	87
7	1,180	856	72·5	57	65
8	1,250	722	58	55	92
9	871	515	59	62	82
	(758)*	(466)	(61·5)		
10	1,910	1,110	58	68	86
11	520	495	95	77	82
	(423)	(420)	(99)		
12	582	246	42	87	95
	(708)	(493)	(69·5)		
Mean..	1,006	750	74·6	63	82

* Figures in parentheses were determined by the method of Euler and Hamberg (1949).

TABLE II

ESTIMATION OF AMINE SYNTHESIS

Cat	Loss of Amine from Left Adrenal (μg./g.)	Estimated Output from Left Adrenal (μg./g.)	Synthesis of Amines (μg./g.)	Synthesis as % of Amine Content of Right Gland
1	400	440	40	6
2	145	360	215	34
3	200	365	165	11
4	240 (gain)	305	545	51
5	170	455	285	20
6	23	335	312	71
7	324	398	74	6
8	528	1,330	802	64
9	356	713	357	41
10	800	1,240	440	23
11	25	238	213	41
12	336	828	492	84
Mean	256	584	328	38

gland was stimulated for a total time of 50 min. the mean output from the gland was 584 μg. Since the mean gland weight was 257 mg. this represented an output of 150 μg./gland in 50 min. or an output of 3 μg./gland/min. If both glands had been stimulated the output would presumably have been 6 μg./cat/min. This figure is somewhat larger but similar to that obtained by Vogt (1952), who stimulated denervated glands of the cat by the intra-arterial injection of KCl and found that the total amount of amines released per min. "was usually of the order" of 4 μg. The figure of 6 μg./cat/min., or 2.4 μg./kg./min., is also in agreement with that of Celander (1954), who, using the same general procedure as in the present series of experiments, gave the figure of 3 μg./kg./min.

The evidence now presented shows that, of the 3 μg./min. liberated from one gland, only 1.3 μg. came from the store in the gland, and 1.7 μg. or 56% was obtained by fresh synthesis. Thus with splanchnic stimulation the two glands of the cat can presumably synthesize 3.4 μg./min., and, taking the average cat weight as 2.5 kg., this is 1.36 μg./kg./min.

Such a result indicates a need to reconsider the conclusion of Hökfelt (1951) and of Udenfriend *et al.* (1953) that the rate of formation of adrenaline is extremely slow. While this may be true under normal conditions when the adrenals are not stimulated, it seems that under the influence of constant impulses from the splanchnic nerves the rate of synthesis may be very much increased, as was suggested by the observations of Tscheboksaroff (1910) and of Elliott (1912), to which reference has already been made. The figure of 1.36 μg./kg./min. is very much larger than the figure of 0.002–0.004 μg./kg./min., mentioned by Udenfriend *et al.* (1953).

The second result of the present experiments was that in every experiment the percentage of adrenaline in the stimulated gland was found to be greater than that in the unstimulated gland, the mean figures being 82% and 63% respectively. This observation is capable of two different explanations. It can be explained by supposing that stimulation released much more noradrenaline than adrenaline from the store in the gland, so that the percentage of adrenaline increased in the store. It can also be explained by supposing that stimulation increased the rate of methylation of noradrenaline. Against the first explanation are the figures of Bülbring and Burn (1949), who found that the proportion of adrenaline to noradrenaline released in the blood stream by splanchnic stimulation varied from a ratio of 98:2 to a ratio of 15:85, with a mean ratio of 59:41. Furthermore Holtz, Engelhardt, Greef, and Schümann (1952) found 80% adrenaline in the amines present in the adrenal vein during splanchnic stimulation. These results give no support to the idea that splanchnic stimulation always releases a greater proportion of noradrenaline than that present in the gland. In favour of the second explanation are the results of Bülbring (1949), who found that the methylation of noradrenaline in adrenal homogenates was much greater if the homogenates were prepared from glands which were stimulated for 30 min. before they were removed from the body. Whereas in homogenates prepared from non-stimulated glands the mean increase in adrenaline was 19%, in homogenates from glands which had been stimulated the mean increase in adrenaline was 60%.

The observations described in this paper thus support the view that the chemical processes in the adrenal gland which underlie the formation of noradrenaline can be greatly accelerated by the stimulation of the splanchnic nerves, and that statements concerning rates of formation in conditions in which there is no stimulation may be inapplicable during splanchnic activity.

Summary

1. The effect of splanchnic stimulation on the synthesis of catechol amines in cat adrenal glands has been investigated.

2. After a stimulation period of 50 min. there was a mean decrease of 256 μg./g. gland and an estimated output of 584 μg. The difference of 328 μg. represents the synthesis. If this value for synthesis is expressed as percentage of what was present in the unstimulated gland it is 38%.

3. The results thus indicate that stimulation liberated 3 μg. of amines from one gland per min., of which 1.3 μg. came from the store in the gland and 1.7 μg. from synthesis. With splanchnic stimulation the two glands of the cat can presumably synthesize 3.4 μg./min. or 1.36 μg./kg./min. This is much higher than figures published for resting glands.

4. It is concluded that splanchnic stimulation increases the rate of synthesis and methylation of noradrenaline.

We wish to thank Professor J. H. Burn for his advice and interest in this work. The work was done by one of us (H. J. S.) during the tenure of a bursary from the British Council.

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THE MODE OF ACTION OF A MIXTURE OF PYRIMETHAMINE AND SULPHADIMIDINE ON *EIMERIA TENELLA*

BY

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It has been suggested by Joyner and Kendall (1955) that a combination of pyrimethamine and sulphadimidine is likely to be of practical value for the control of caecal coccidiosis caused by *Eimeria tenella* in chicks. Further knowledge of its mode of action, and the phases of the life history of the parasite that are most susceptible, is therefore desirable.

The development of *Eimeria tenella* includes two schizogonous cycles before the formation of gametocytes and oocysts. Studies by Horton-Smith and Taylor (1945) and Horton-Smith and Boyland (1946) have shown that sulphonamides inhibit the second generation schizonts by interference with *p*-aminobenzoic acid metabolism. The extension of the work of Wehr and Farr (1947) by Kendall and McCullough (1952) has shown that for satisfactory therapy this is the only stage which should be controlled. Inhibition of earlier stages, which occurs with certain doses of sulphonamides, leads to incomplete protection, and there are further deaths after stopping the drug. In the early experiments with pyrimethamine and sulphadimidine (Joyner and Kendall, 1955) there was some evidence that the mixed drugs might exert a specific effect upon the second generation schizonts.

The experiments described in this paper were designed to investigate this point in greater detail and to attempt an assessment of the role of the two drugs as inhibitors of the two metabolites—*p*-aminobenzoic acid and folic acid—which they are believed to affect (Goodwin and Rollo, 1955).

METHODS

Rhode Island Red or White Leghorn chicks, reared in isolation from adventitious infection, were inoculated orally with sporulated oocysts of *E. tenella* in numbers sufficient to kill at least 70% of untreated birds. The chicks, which were 1 to 3 weeks old, were kept in heated wire-floored brooders and allowed unlimited food and water. The diet was composed of a proprietary chick mash in which the drugs were incorporated during the experimental period. The food-drug mixtures were prepared with the aid of a mechanical mixer.

Folic acid and *p*-aminobenzoic acid were given twice daily, by intraperitoneal injection. The first dose was given 5 hr. after beginning therapy, and the second dose 5 hr. after ending it.

RESULTS

The Effect Upon Different Stages of the Parasite.—The development of *E. tenella* follows a regular course after infection with a single dose of oocysts. The first schizogony starts at about the 48th hour and the second shortly after the 72nd hour. It follows that by beginning drug treatment at different intervals after infection, and observing the effects upon the development of the disease, an indication may be obtained of the relative susceptibility of the different developmental stages of the parasite.

TABLE I

EFFECT OF TREATMENT AT DIFFERENT STAGES OF *E. TENELLA* INFECTION IN CHICKS

A mixture of 0·004% pyrimethamine and 0·05% sulphadimidine was given in the food at different stages in the development of *E. tenella* infection. All groups, each of 14 chicks, were infected on day "0." Drug treatments covered successive periods of 3 days, from 3 days before infection (Group A) to 7 days after (Group H). The deaths in each group are tabulated

Day after Infection	Treatment with Drugs on Days:								
	−3 to 0 A	−2 to 1 B	−1 to 2 C	0 to 3 D	1 to 4 E	2 to 5 F	3 to 6 G	4 to 7 H	Controls J
5	5	3					1	6	6
6	1	3	7	1				1	3
7			1	3					1
8				2					
9					1				
Total deaths	6	6	8	6	1	0	1	7	10

In the experiment recorded in Table I, 8 groups of 14 chicks were given 3-day courses of treatment with a mixture of 0.004% pyrimethamine and 0.05% sulphadimidine in the food; treatment began at different times during the development of the infection. A further group was kept untreated as a control.

Mortality was relatively unaffected when treatment ended on the day of infection, or was delayed until the fourth day. In both these groups of

chicks (A and H), as in the control group (group J), the greatest number of deaths occurred on the fifth day.

Deaths were progressively delayed in groups B, C, and D when therapy was stopped 1, 2, and 3 days after infection respectively. Almost complete control of the infection was obtained in groups E, F, and G when treatment ended 4, 5, and 6 days after infection.

It may be concluded that treatment given during the first 72 hr. retards the development of the early schizonts, whereas the effect upon the later schizonts is lethal. This is in agreement with the preliminary studies reported by Kendall (1956).

Inhibition by Metabolites.—Studies with other micro-organisms have led to the conclusion that pyrimethamine interferes with the conversion of folic to folinic acid, and in some organisms its action can be reversed by simultaneous administration of either of these metabolites (Goodwin and Rollo, 1955).

As the action of sulphadimidine on *E. tenella* can be reversed by *p*-aminobenzoic acid (Horton-Smith and Boyland, 1946) we decided to compare the inhibitory effects of folic acid and *p*-aminobenzoic acid against pyrimethamine and sulphadimidine given either separately or in combination.

Table II shows that both metabolites antagonized pyrimethamine. In experiment A, 0.01% pyrimethamine was completely antagonized by 100 mg./kg. folic acid. With the higher dose of the drug, however, neither metabolite caused complete antagonism.

TABLE II

ANTAGONISM OF THE THERAPEUTIC EFFECT OF PYRIMETHAMINE AND SULPHADIMIDINE ON *E. TENELLA* BY FOLIC ACID AND *p*-AMINOBENZOIC ACID

Expt.	Metabolite Given by Injection	Mortality, % with					
		No Drug	Pyrimethamine in the Food (%)		Sulphadimidine in the Food (%)		
			0·01	0·02	0·1	0·2	0·4
A	Folic acid: 100 mg./kg.	90	92	25	—	—	—
	p-Aminobenzoic acid:- 100 mg./kg.	100	—	40	83	100	92
	25 ,, ..	—	—	30	75	83	45
	Nil	80	33	8	42	0	0
B	Folic acid: 100 mg./kg.	—	50	33	75	33	17
	50 ,, ..	—	67	42	83	42	—
	p-Aminobenzoic acid: 100 mg./kg.	—	83	42	—	—	—
	25 ,,	—	92	17	—	—	—
	Nil	100	67	8	67	0	0

The antagonism by *p*-aminobenzoic acid was marked even with relatively high doses of sulphadimidine—well above those which in this series of experiments were required for complete protection against *E. tenella* infection. With folic acid there was some antagonism of the action of the sulphonamide, but much less than with *p*-aminobenzoic acid.

It was not possible from the data obtained with pyrimethamine to decide whether the action of either metabolite was competitive, but with sulphadimidine there was evidence for competition by both folic acid and *p*-aminobenzoic acid. For example, in experiment A, with 0.4% sulphadimidine in the food, a reduction in the dose of *p*-aminobenzoic acid from 100 mg./kg. to 25 mg./kg. resulted in a reduction in the degree of antagonism. With doses of *p*-aminobenzoic acid of 25 mg./kg. in the same experiment, antagonism was reduced when the amount of sulphadimidine in the food was increased from 0.2% to 0.4%. In experiment B, with folic acid in a dose of 100 mg./kg. the degree of inhibition varied inversely with the amount of sulphadimidine administered.

TABLE III

ANTAGONISM OF THE THERAPEUTIC EFFECT OF MIXTURES OF PYRIMETHAMINE AND SULPHADIMIDINE ON *E. TENELLA* BY FOLIC ACID

Experiment No.:	1	2	3	4
Pyrimethamine in food (%)..	0·004	0·004	0·004	0·005
Sulphadimidine ,, ,, ,, ..	0·05	0 05	0·04	0·05
	% Mortality			
Folic acid 100 mg./kg. ..	8	0	61	60
No metabolite	0	0	13	0
Untreated control	75	100	73	92

Synergism between sulphadimidine and pyrimethamine was apparent when relatively small doses of the two drugs were given together. Thus in the experiment illustrated in Table III, in which mixtures of sulphadimidine and pyrimethamine were given, almost complete therapeutic control was obtained with doses between one-fifth and one-eighth of those used to obtain the same effect in the experiments recorded in Table II. It might be expected, therefore, that correspondingly lower doses of the metabolites would be necessary to antagonize the action of the drugs—that is, the synergistic combination might be more readily antagonized by the metabolites.

Antagonism of mixtures of pyrimethamine and sulphadimidine using large doses of folic acid proved, however, to be incomplete and irregular, as shown in the four experiments recorded in Table III. Only in experiments 3 and 4 was the

percentage mortality appreciably raised by the administration of folic acid simultaneously with the drugs.

Antagonism of the effects of the mixture by *p*-aminobenzoic acid occurred more regularly, but as much of the metabolite was required when the mixture was used as when large doses of sulphadimidine were given alone. This is demonstrated in Table IV, where groups of chicks were given either 0.3% sulphadimidine, or a mixture of 0.05% sulphadimidine and 0.004% pyrimethamine. Both treatments gave complete protection (A, Table IV), and increasing doses of *p*-aminobenzoic acid progressively reduced the effects of treatment (B, C, and D).

The dose of *p*-aminobenzoic acid required to increase the mortality with either drug treatment to one half of that in the untreated controls (E, Table IV) was estimated by plotting the results

TABLE IV

ANTAGONISM ON *E. TENELLA* OF THE THERAPEUTIC EFFECT OF SULPHADIMIDINE, ALONE AND IN COMBINATION WITH PYRIMETHAMINE, BY *p*-AMINOBENZOIC ACID

Group	*p*-Aminobenzoic Acid Injected Twice Daily mg./kg.	% Mortality	
		0·3% Sulphadimidine	0·05% Sulphadimidine + 0·004% Pyrimethamine
A	0	0	0
B	10	25	8·3
C	25	41·6	16·6
D	50	66·7	58·3
E	Untreated control mortality, 83%		

graphically. It was found that 25 mg./kg. of *p*-aminobenzoic acid was required to produce this effect when 0.3% sulphadimidine alone was given. Similarly, 42.5 mg./kg. *p*-aminobenzoic acid was required when 0.05% sulphadimidine was given together with 0.004% pyrimethamine.

It was concluded that folic acid was a weak antagonist of both pyrimethamine and sulphadimidine. *p*-Aminobenzoic acid was an active antagonist of sulphadimidine, but resembled folic acid in having only a slight inhibitory effect upon pyrimethamine (Table II). When reduced doses of the two drugs were given together, there was no apparent increase in sensitivity to the antagonistic effects of the metabolites (Tables III and IV).

DISCUSSION

It is evident from Table I that the combination of low doses of pyrimethamine and sulphadimidine affects the development of *E. tenella* in the same way as the other effective drugs so far investigated —sulphaquinoxaline (Cuckler and Ott, 1947), nitrofurazone (Horton-Smith and Long, 1952), and sulphadimidine itself (Horton-Smith and Taylor, 1945).

The sensitivity of the late schizonts is probably due to several physiological factors. The intensely active division in this stage of the life-cycle of *E. tenella* must result in extreme susceptibility to any metabolic deficiency induced by drug action. If the difference in susceptibility to drugs of the early and late schizonts is due to quantitative differences in metabolic activity, then the earlier stages, which divide less actively, would be expected to be less sensitive. This, in fact, has been shown to occur (see Table I). It is also possible that qualitative metabolic differences exist; the different location of the first and second generation schizonts in the caecal tissue, and the different course of development of the resultant merozoites, lend support to this possibility.

The results of experiments with drugs and antagonists can provide only circumstantial evidence for the existence of metabolic reactions. The results of the experiments with folic acid and *p*-aminobenzoic acid described in this paper do, however, suggest similarities between the metabolism of *E. tenella* and plasmodia, to which they are systematically related.

Experiments with a number of organisms have provided evidence for the belief that pyrimethamine interferes with the conversion of folic to folinic acid (Hitchings, 1952). The success with which pyrimethamine can be antagonized by these metabolites seems to depend upon whether or not the organism ordinarily synthesizes them within the cell. The effect of pyrimethamine on *Streptococcus faecalis*, which utilizes exogenous sources of folinic acid, is easily reversed by the metabolite.

In the malarial parasite, folic and folinic acids have only a weak effect upon the action of pyrimethamine. It has been suggested, therefore, that the malarial parasite synthesizes folic acid within the cell, and that the preformed metabolite is not available to it (Goodwin and Rollo, 1955). Experiments with *E. tenella* have shown that with this parasite also, folic acid has only a slight effect upon the activity of pyrimethamine. Therefore, like the plasmodia, *E. tenella* probably is unable to utilize preformed folic acid. It is likely that the slight antagonistic effect of folic acid upon sulphadimidine is due to the breakdown within the chick of folic acid to *p*-aminobenzoic acid. Rollo (1955) has suggested this for *Plasmodium gallinaceum*, where a similar phenomenon has been observed.

The slight antagonistic activity of *p*-aminobenzoic acid against the action of pyrimethamine

on *E. tenella* is in agreement with the experience of Thurston (1954) with *P. berghei*, and of Rollo (1955) with *P. gallinaceum*, but it has not been possible to conclude from the data presented above that the antagonism with *E. tenella* is competitive.

The comparison of the antagonism of *p*-aminobenzoic acid to sulphadimidine alone, and to mixtures of this sulphonamide and pyrimethamine, is of considerable interest. Both drug treatments give almost complete control of the infection, but when used together the amount of the sulphonamide required is very much reduced. It would therefore be expected that much smaller amounts of *p*-aminobenzoic acid would be required to antagonize the combination. Table IV shows that, on the contrary, almost the same dose of *p*-aminobenzoic acid is required to antagonize either 0.3% sulphadimidine or 0.05% sulphadimidine together with 0.004% pyrimethamine.

It has not so far proved possible to induce any marked degree of drug-resistance in *E. tenella* which would provide further information on the mode of action of drugs (Goodwin and Rollo, 1955). Theories of drug action must therefore remain conjectural and based mainly on analogy with other organisms. All the evidence so far available indicates that pyrimethamine and sulphonamides act upon the second generation schizonts of *E. tenella* in the same way as they act upon the schizonts of some species of *Plasmodium*.

Summary

1. A mixture of 0.004% pyrimethamine and 0.05% sulphadimidine in the food was administered to chicks at different periods after infection with *E. tenella*. Treatment of the early schizonts earlier than the 72nd hr. after infection resulted in a retardation of development. Treatment of the later schizonts resulted in complete suppression.

2. Antagonism of the effects of pyrimethamine and sulphadimidine was studied by the simultaneous administration of either folic acid or *p*-aminobenzoic acid. Folic acid was a weak antagonist of therapeutic amounts of both pyrimethamine and sulphadimidine. *p*-Aminobenzoic acid was an active antagonist of sulphadimidine, but resembled folic acid in having only a slight inhibitory effect upon pyrimethamine. When reduced doses of both drugs were given there was no apparent increase in the inhibitory effects of the two metabolites.

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EFFECT OF THIOPENTONE ON BLOOD SUGAR AND GLUCOSE TOLERANCE

BY

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Attention has recently been drawn by Hunter and Greenberg (1954) and Merivale and Hunter (1954) to the abnormal blood sugar response to glucose that occurs in patients who are receiving large doses of sedative and hypnotic drugs, particularly barbiturates. All types of glucose tolerance curves have been reported, and, as yet, no really satisfactory explanation for the abnormalities has been found.

Similar disturbances in glucose metabolism have been reported by Booker and his associates (1946, 1949) in dogs following thiopentone administration. In animals on normal diets liver glycogen was found to be progressively depleted during long anaesthesia, and the administration of glucose before the induction of anaesthesia produced hyperglycaemia and glycosuria. Even animals whose livers were depleted of carbohydrate by starvation, before the administration of thiopentone, were unable to convert glucose to glycogen, and remained hyperglycaemic. Intermediate metabolism of carbohydrates was also depressed by prolonged thiopentone anaesthesia, as shown by a rise in the blood lactic acid content. All these changes could be mitigated by the use of small doses of insulin, if given along with or immediately following the administration of the thiopentone. Other workers (Blackberg and Hrubetz, 1937; Hrubetz and Blackberg, 1938; Richards and Appel, 1941) have also reported finding a mild hyperglycaemia during thiopentone anaesthesia in animals.

No significant changes in blood sugar were noted during thiopentone anaesthesia in man by Carraway (1939) or by Sessoms, Watts, Chase, and Andrews (1955). Other workers (Cameron, 1937; Thomas, 1938; Marshall, 1939; Ruth, Tovell, Milligan, and Charleroy, 1939) found a transitory slight hyperglycaemia returning to normal within a few hours of return of consciousness. Sessoms *et al.* (1939) found that patients receiving thiopentone–nitrous oxide–oxygen behave in a similar manner to those receiving thiopentone alone. It has also been noted by Bass, Watts, and Chase (1953) that ether hyperglycaemia is inhibited by induction of anaesthesia with thiopentone.

Stern, Papper, Bueding, and Rovenstine (1945) have studied glucose tolerance in three subjects who were anaesthetized for 45 to 60 min. with thiopentone. They found that the intravenous administration of 1.5 g. of 50% glucose produced a greater rise in blood sugar than when the same amount was administered in the conscious state. Their findings suggest a similarity in behaviour of glucose tolerance in man and dogs during thiopentone anaesthesia. However, these observations are open to criticism in that all three subjects were admitted to hospital with acute alcoholism, since hyperglycaemic responses are also found in chronic alcoholics (Bowman, Wortis, Orenstein, and Goldfarb, 1939).

This paper describes observations made in man on blood sugar changes during thiopentone anaesthesia with and without operations. Changes in the glucose tolerance curve produced by thiopentone have also been studied in healthy subjects. A preliminary mention of some of this work has appeared elsewhere (Dundee, 1956).

METHODS

This study was carried out on eight healthy adult volunteer subjects (6 males, 2 females), aged 23–51 years. These were admitted to hospital for ligation of varicose veins. Observations were made as follows:

(a) Blood sugar determinations at 30 min. intervals, during prolonged administration of thiopentone.

(b) Blood sugar determinations at 10–20 min. intervals, during operation for ligation of veins. Anaesthesia was with thiopentone–nitrous oxide–oxygen, and morphine 10 mg. with atropine 0.6 mg. was given as pre-operative medication.

(c) Glucose-tolerance test, no anaesthesia; 50 g. glucose in 150 ml. water was given by mouth and blood samples drawn at approximately 30, 50, 70, 100, and 130 min. thereafter (six subjects).

(d) Glucose-tolerance test under thiopentone anaesthesia. Glucose given as above and anaesthesia induced 10 min. later and continued for a period of 100–270 min. This was done on the same six subjects as in (c).

All patients had fasted for 3–4 hr. before each procedure save (b), when the time from the last meal varied from 4 to 11 hr. The blood sample for the control blood sugar reading was withdrawn immediately before the administration of the thiopentone in (a) and (b) and before the glucose in (c) and (d). The order in which the above investigations were carried out varied in each case, but the interval between each administration of thiopentone was always longer than one week. The normal response to glucose (c) was estimated at different times, depending on circumstances—on two occasions on the day before operation; in three subjects during the second week after operation and once about six weeks after the patient had left hospital.

All anaesthetics were given personally and all administrations were smooth throughout with no marked respiratory depression or hypotension. As far as possible the same dose of thiopentone was given on each occasion, but in no instance was the amount of drug given more than appeared necessary to maintain the desired degree of narcosis. The doses of thiopentone which, following pre-operative medication and combined with 6 l. nitrous oxide and 2 l. oxygen/min., produced satisfactory operating conditions, if given alone were sufficient to produce light narcosis with the occasional return of the corneal reflex. There were no untoward sequelae following any of the anaesthetics and convalescence after operation was uneventful, in all cases.

Blood sugar estimations were carried out by the method described by Folin and Wu (1930), using venous blood from the forearm. For repeated withdrawals of blood and injections of thiopentone the Mitchell self-sealing needle (Mitchell, 1952) proved very useful.

Results

Table I gives the average blood sugar readings and doses of thiopentone given in investigation (a) when no operation was carried out or other drugs given. At no time was the increase in blood sugar level significantly different from the control reading.

The average effects on blood sugar of thiopentone–nitrous oxide–oxygen after pre-operative morphine and atropine in subjects undergoing non-abdominal surgery are shown in Table II. These show a significant increase in glucose content of the blood samples drawn between 5 and 50 min. after induction of anaesthesia. The rise in blood sugar is not very great and its magnitude is decreased if one allows for a possible error of ± 5 mg./100 ml. in the technique of estimation. However, despite this, the rise in blood sugar immediately after the induction of anaesthesia was significantly greater ($P<0.01$) than occurred in the same subjects after thiopentone alone.

Fig. 1 shows the average results in the six patients in whom all four investigations were carried out. The blood sugar readings with and without operation are essentially the same as in Tables I and II and require no further comment. This figure shows that thiopentone produces a marked effect on the blood sugar response to the oral administration of 50 g. glucose. The hyperglycaemic response produced by thiopentone was consistent in all cases, and an analysis of the average increases in blood sugar (Table III) shows the effect of thiopentone to be statistically significant. Urine samples collected in three subjects reduced Benedict's solution after the administration of thiopentone, whereas this only occurred in one of the three subjects when they did not receive any anaesthetic.

Table I

AVERAGE BLOOD SUGAR READINGS IN 8 VOLUNTEERS WHO RECEIVED THIOPENTONE IN DOSES STATED BELOW BUT WHO WERE NOT SUBJECTED TO ANY OPERATION

Time in Minutes	No. of Observations	Average Dose of Thiopentone		Average Blood Sugar (mg./100 ml.)	Average Deviation from Control
		mg.	mg./kg.		
Control	8			84	
20–40	8	769 (450–1,750)	10·7	85	+0·8±4·0
50–70	8	850 (700–1,750)	16·1	84	0
80–100	6	1,228 (825–2,000)	19·5	83	+1·7±1·8
110–130	6	1,560 (900–2,800)	23·8	80	−1·7±1·4
140–160	5	1,700 (1,000–2,800)	26·7	82	0
200–220	4	2,212 (1,200–3,400)	30·1	80	−2·5±1·6
230–250	4	2,306 (1,325–3,400)	35·2	81	−1·2±1·2
260+	3	1,967 (1,400–2,900)	30·4	80	0

Table II

AVERAGE BLOOD-SUGAR READINGS AND DOSES OF THIOPENTONE IN 8 PATIENTS WHO WERE OPERATED ON FOR VARICOSE VEINS UNDER THIOPENTONE-NITROUS OXIDE-OXYGEN ANAESTHESIA

Time in Minutes	No. of Observations	Average Dose of Thiopentone		Average Blood Sugar (mg./100 ml.)	Average Deviation from Control
		mg.	mg./kg.		
Control	8			77	
5–15	8	950 (500–1,400)	13·6	92	+25·3±4·0
20–35	8	1,170 (850–1,700)	16·4	85	+8·3±3·6
50–70	7	1,143 (1,000–1,700)	16·2	88	+10·6±4·7
80–100 (awake)	8	1,350 (1,000–1,700)	19·5	75	−1·8±0·8

FIG. 1.—Average blood sugar readings on six subjects. ——— (upper curve) glucose tolerance test, no anaesthesia; – – – glucose tolerance test, thiopentone anaesthesia; ..... thiopentone anaesthesia alone; ——— (lower curve) thiopentone–nitrous oxide–oxygen anaesthesia: atropine and morphine as pre-operative medication. Operation for varicose veins.

TABLE III

AVERAGE INCREASE IN BLOOD SUGAR AFTER 50 G. GLUCOSE BY MOUTH IN 6 SUBJECTS WITH AND WITHOUT THIOPENTONE ANAESTHESIA

Time after Administration of Glucose (Minutes)	Average Deviation of Blood Sugar Level (1 mg./100 ml.) from Control Value			
	No Anaesthesia (A)	Thiopentone (B)	Difference (A–B)	"t" Value
30	+48± 7·5 (18–100)	+110±10·8 (80–140)	62±13·4	4·7
50	+65± 9·7 (30–100)	+125± 9·2 (90–140)	60±15·0	4·0
70	+57±10·6 (40–80)	+78± 9·3 (40–110)	21±14·5	1·5
100	+27± 5·3 (10–50)	+52± 4·1 (35–80)	25± 6·8	3·7
130	+7± 1·9 (0–20)	+35±7·6 (0–60)	28± 8·0	3·5

DISCUSSION

The number of subjects used in this study is small; but, as they were willing to submit to repeated administrations of thiopentone, it is hoped that the results will be more valuable than would have been obtained from single observations on a larger number of subjects. One cannot be certain from the few results in Table I that thiopentone alone has no effect on blood sugar, but a comparison of Tables I and II shows that the combination of thiopentone with either morphine, atropine, nitrous oxide or operative stress produces a temporary mild hyperglycaemia.

The work of Sessoms *et al.* (1955) shows that, in the absence of hypoxia, nitrous oxide was not the cause of the hyperglycaemia. Factors other than the anaesthetic agents can cause a rise in blood sugar and these have recently been exhaustively reviewed by Foster and Francis (1955). Morphine, despite its depressant effect on the central nervous system, may raise blood sugar and can act as a "stressor" (Selye, 1950). Atropine has been shown by several workers to block insulin secretion produced by vagal stimulation (Portis, 1950; Portis and Zitman, 1943). However, Bass *et al.* (1953) and Goodman and Gilman (1955) consider the effects of therapeutic doses of morphine and atropine to be negligible. The alarm reaction, described by Selye (1950) as part of the body's response to stress, following the initial trauma of surgery may have caused a transient rise in blood sugar, produced by liberation of adrenaline. If marked apprehension had been present before induction of anaesthesia the control blood sugar reading in Table II should have been raised, whereas it was lower than before any of the other investigations.

A more likely explanation for the rise in blood sugar during operation is the combined respiratory depressant effects of thiopentone and morphine, which Eckenhoff and his colleagues (Eckenhoff *et al.*, 1954, 1955; Helrich *et al.*, 1956) have shown to be much more marked than that produced by thiopentone alone. Hypercarbia and hypoxia can both raise the blood sugar level; in fact, these are the reasons given by Goodman and Gilman (1955) for the hyperglycaemia that follows large doses of morphine.

Save for the report of Sessoms *et al.* (1955) sufficient data on dosage of thiopentone and nature of operations, etc., are not given to allow the results of this investigation to be compared with those of other workers. Sessoms found an insignificant drop in the blood sugar levels of patients undergoing dilatation of the cervix and curettage of the uterus under thiopentone alone or under thiopentone–nitrous oxide–oxygen anaesthesia. The operations are comparable in that neither involves the peritoneal cavity or autonomic nervous system. Although the doses of drugs used as pre-operative medication are not given in Sessoms' paper, they are unlikely to have differed greatly from those given before operation in this study. However, the average doses of thiopentone given by Sessoms and his colleagues were 675 (575–800) mg. when the drug was given alone and 463.8 (300–750) mg. when it was combined with nitrous oxide–oxygen.

The latter figure is approximately half that used for the first 5 to 15 min. in the operated patients in this study (Table II), and the consequently greater degree of respiratory depression is the most probable explanation for the different results obtained.

The altered glucose tolerance in patients receiving thiopentone (Fig. 1 ; Table III) is in full agreement with the findings of Booker (1946) in dogs and Stern *et al.* (1945) in man. It has been shown (Booker, 1946 ; Booker, French, and Molano, 1949) that intermediate metabolism of carbohydrates is also interfered with by thiopentone, and these workers consider the depression of the glycogenolytic-glycogenic activity of the liver by thiopentone to be indirect evidence of the role of this organ in its metabolism. The hepatic detoxication of thiopentone is now proved beyond all reasonable doubt (Shideman, Kelly, and Adams, 1947 ; Shideman, Kelly, Lee, Lovell, and Adams, 1949 ; Walker and Wynn Parry, 1949 ; Dundee, 1952 ; Meyers and Peoples, 1954), and it seems more likely that this is a manifestation of the hepatic toxicity of large doses of thiopentone, since the author (1955) has shown that similar doses interfere with other functions of the liver.

Summary

1. Thiopentone anaesthesia alone produces no appreciable effect on the blood-sugar levels in man.

2. Slight hyperglycaemia was observed when thiopentone–nitrous oxide–oxygen anaesthesia, following morphine and atropine as pre-operative medication, was used as anaesthesia for operations on varicóse veins.

3. The rise in blood sugar during operation differed significantly from that produced by the anaesthetic alone, and may be secondary to respiratory depression from the morphine–thiopentone combination.

4. Thiopentone markedly depresses the ability of the body to deal with an extra load of glucose.

5. The depression in glucose tolerance is thought to be a manifestation of the hepatotoxic effect of large doses of thiopentone.

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THE URINARY EXCRETION OF SUBCUTANEOUSLY INJECTED HISTAMINE

BY

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Histamine is excreted in the urine in a conjugated form, derived from the alimentary tract, and in a free form, derived mainly from endogenous sources (Anrep, Ayadi, Barsoum, Smith, and Talaat, 1944; Adam, 1950). When histamine is given by intravenous infusion to healthy human beings, about 1% of the dose appears in the urine as free histamine (Adam, 1950; Adam, Card, Riddell, Roberts, and Strong, 1954). These reports suggested that a similar technique might be used to determine how histamine entering the blood stream is dealt with under abnormal conditions. The intravenous infusion of histamine, however, presents practical disadvantages which limit its usefulness as a clinical test. Anrep and his colleagues (1944) showed that the subcutaneous injection of histamine in dogs resulted in the appearance of free histamine in the urine, and the present work was therefore undertaken to gain more information about the excretion of histamine administered to human beings by subcutaneous injection.

METHODS

Free histamine was extracted from urine by the method of Roberts and Adam (1950). The dried, acidified extracts were taken up in 5 ml. of warm Tyrode solution, neutralized, and assayed on a strip of guinea-pig ileum suspended in a bath of Tyrode solution containing atropine (0.1 μg./ml.), using a standard solution of histamine acid phosphate for comparison. A series of 10 recovery experiments, in which histamine was added to urine before extraction at the rate of 1 μg./50 ml. (5 tests) or 5 μg./50 ml. (5 tests), gave a mean recovery of 63.4% with a standard error of 2.8, which is not significantly different ($P > 0.3$) from the mean recovery of 66.5% ± 1.8 reported previously (Mitchell and Code, 1954a). The figure of 63.4% has been applied as a correction factor in the present work, but all values for histamine are given uncorrected in terms of histamine base, unless stated otherwise.

Evidence that the activity measured was due to histamine was obtained by frequent tests with mepyramine maleate (Roberts and Adam, 1950), and occasional parallel assays on the cat's blood pressure, when satisfactory agreement was always obtained.

Basiphil and eosinophil leucocytes in capillary blood were enumerated in the same counting chamber according to the method of Moore and James (1953).

The experiments were carried out on healthy men aged between 26 and 34 years. In a preliminary experiment, a meal of 500 g. of beef steak was given to a man who had fasted for 24 hr., and the urinary free histamine was estimated during each period of 2 hr. for 4 hr. before and 14 hr. after the meal.

Before each of the subsequent experiments, the subject fasted for at least 10 hr. At 5.30 a.m., urine was voided and discarded; thereafter urine was collected at the end of each 2-hr. period by voiding directly into a bottle containing 0.5 ml. of 2N-HCl. The experiment was completed at 1.30 p.m., giving four urine specimens for extraction and assay of histamine. The first and second gave values for the basal excretion of free histamine under fasting conditions; during the third collection period, histamine acid phosphate was injected subcutaneously, the total dose being injected into the thighs in four equal parts, at 9.30, 10, 10.30 and 11 a.m. Throughout the 8 hr. of the experiment, the subject continued to fast but drank 120 ml. of water each time he voided urine, to ensure an adequate output of urine.

The eosinophils and basiphils in the blood were counted before, during and after the 2-hr. period during which the histamine was given, at 9.25, 10.30 and 11.35 a.m.

At least four weeks elapsed between successive experiments on any individual. Three experiments were performed on each of four subjects (A, B, C, and D). In the first experiment of each series, a total dose of 5 μg. of histamine acid phosphate for each kg. of body weight was injected, in the second 10 μg./kg., and in the third 20 μg./kg.

One experiment was carried out (on subject A) without any change in the above details except for the substitution of sterile normal saline for histamine in the injections.

In a further experiment, a healthy man was given 200 mg. of cortisone acetate by mouth, and the basiphils and eosinophils in the blood were counted at hourly intervals for 2 hr. before and 11 hr. after administration. Two-hourly specimens of urine were

TABLE I

THE EXCRETION OF FREE HISTAMINE IN THE URINE OF FOUR HEALTHY MEN BEFORE, DURING AND AFTER THE SUBCUTANEOUS INJECTION OF HISTAMINE ACID PHOSPHATE

(Given in four doses over 2 hr.)

Total Dose (μg./kg.) of Histamine Acid Phosphate Injected During Period III	Subject and Body Weight (kg.)	Free Histamine in Urine in μg. Histamine Base/2-hr. Period				Extra Histamine Excreted in Period III as % of Total Dose Injected
		I 5.30–7.30	II 7.30–9.30	III 9.30–11.30	IV 11.30–1.30	
5	A. 70	0·43	0·37	0·83	0·54	0·54
	B. 70	0·77	1·07	1·28	1·06	0·45
	C. 84	0·96	—*	1·62	0·68	0·69
	D. 68	0·77	0·68	1·05	0·79	0·42
	Mean	0·73	0·71	1·20	0·77	0·53
10	A. 70	0·60	0·53	2·68	0·74	1·32
	B. 70	0·62	0·72	2·48	1·12	1·13
	C. 84	1·03	0·99	3·04	1·14	1·05
	D. 70	0·75	0·84	2·33	0·47	0·96
	Mean	0·75	0·77	2·63	0·87	1·12
20	A. 70	0·67	0·39	5·06	0·88	1·41
	B. 70	0·78	0·63	3·94	0·90	1·01
	C. 84	1·10	0·95	5·04	1·72	1·17
	D. 75	0·43	0·56	5·04	0·61	1·32
	Mean	0·75	0·63	4·77	1·03	1·23

* Sample lost.

(Note: The weight of subject D increased in the several weeks between each experiment.)

collected for free histamine determination for 2 hr. before and 8 hr. after giving the hormone. The man fasted for 24 hr. before the experiment and throughout its duration. This experiment was repeated on the same subject using 50 mg. of corticotrophin in saline solution given by intramuscular injection, instead of cortisone.

In all the experiments, the extraction of histamine was started within a few hours of completing the final collection of urine.

RESULTS

In the preliminary experiment the amount of free histamine in the urine started to increase

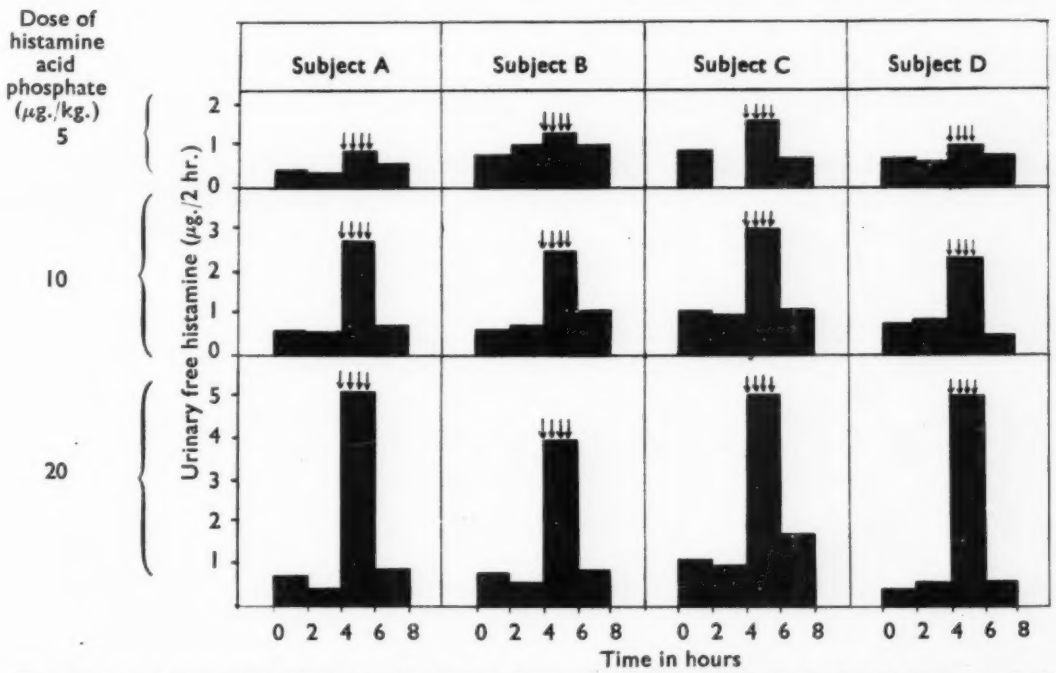

FIG. 1.—The effect of subcutaneously-injected histamine on the urinary excretion of free histamine by four healthy men. Total dose of histamine acid phosphate injected in four doses as indicated by the sets of four arrows.

within two hours of the meat meal, increased rapidly to a maximum of 2.0 μg. during the next two-hour period and returned to the fasting level of 0.5 μg./two hours at 10 hours after the meal. This pattern of free histamine excretion after a meat meal has also been found in dogs and in other human subjects (Irvine, 1956).

The changes in urinary free histamine following the subcutaneous injection of histamine are shown in Table I and Fig. 1. During the first and second two-hour periods the amount of free histamine excreted was small, ranging from 0.37 μg. to 1.10 μg./two-hour period, the mean value of 0.72 μg. being slightly less than the value of 1.05 μg. (range 0.44 to 1.67 μg.) calculated from the data of Adam and his colleagues (1954). When saline was injected during the third period, the rate of histamine excretion remained approximately constant, the amounts excreted in the four periods being 0.50, 0.40, 0.56, and 0.53 μg.

In all 12 experiments in which histamine was injected during the third two-hour period, the amount of free histamine in the urine increased above the fasting level during that period, and in every instance it decreased again in the fourth period, usually though not always to the fasting level (Table I). All, or almost all, of the extra free histamine was therefore contained in the third urine specimen, and in the calculations the small amounts occasionally excreted during the fourth period have been disregarded. Hence in each case the mean value for free histamine in the first two specimens was subtracted from the value in the third specimen to obtain the value for the extra histamine excreted. In the calculations, this value is multiplied by $\frac{100}{63.4}$ (the correction factor for the Decalso method) and then converted to the equivalent value for histamine acid phosphate, which is assumed to contain 36.16% of histamine base. The result is expressed as a percentage of the total dose of histamine acid phosphate injected.

The calculations show that when 5 μg. of histamine acid phosphate/kg. of body weight was injected subcutaneously during a period of two hours, 0.53% of the dose was excreted during the same period. This mean rate of excretion increased to 1.12% when the total dose was doubled to 10 μg./kg., and to 1.23% when the dose was doubled again to 20 μg./kg. (Table I).

Table II shows that there was no consistent change in the numbers of circulating basiphils or

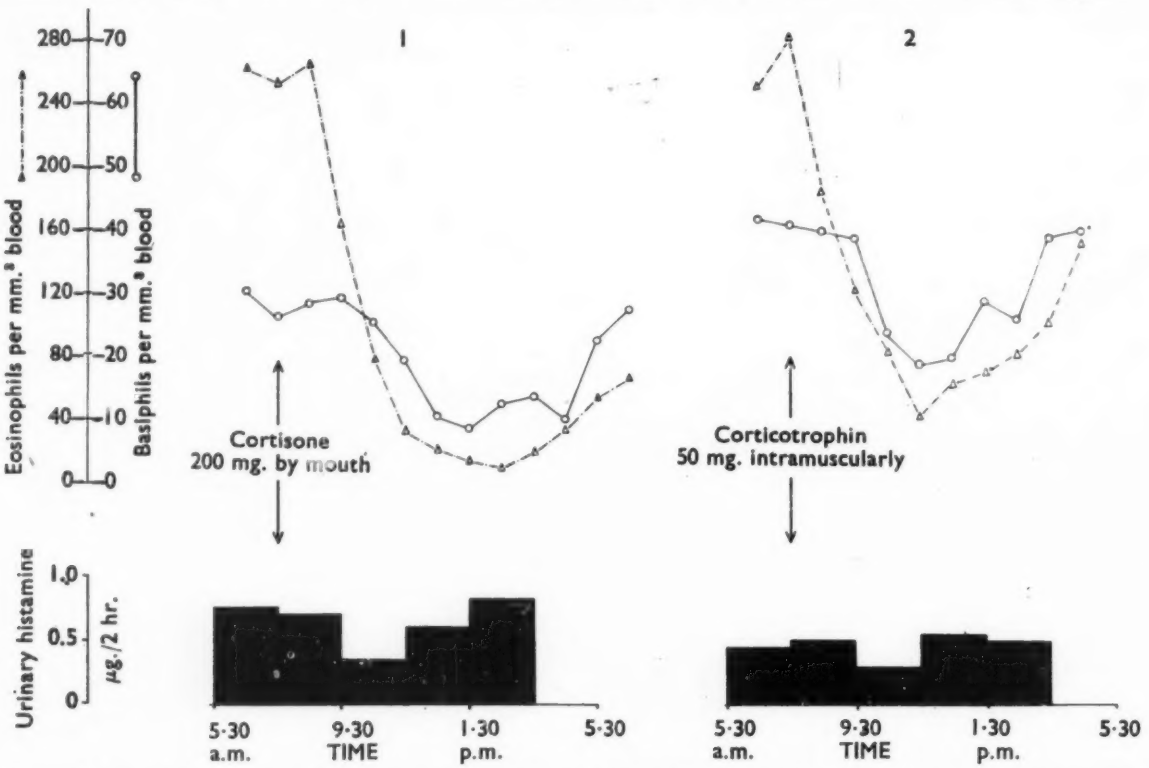

FIG. 2.—The effect of cortisone and of corticotrophin on the numbers of eosinophils and basiphils in the blood, and on the amount of free histamine in the urine of a fasting man (age 34 yr., wt. 75 kg.).

TABLE II

NUMBERS OF BASIPHIL AND EOSINOPHIL LEUCOCYTES IN THE BLOOD OF FOUR HEALTHY MEN BEFORE, DURING AND AFTER THE SUBCUTANEOUS INJECTION OF HISTAMINE ACID PHOSPHATE

(Given in four doses over 2 hr.)

Dose of Histamine Acid Phosphate (μg./kg.)	Subject	No. of Basiphils per c.mm. of Capillary Blood			No. of Eosinophils per c.mm. of Capillary Blood		
		Before	During	After	Before	During	After
5	A	22	20	19	72	81	69
	B	56	61	57	313	256	254
	C	41	40	36	300	344	276
	D	22	20	22	207	210	162
	Mean	35	35	34	223	223	190
10	A	19	26	14	141	85	62
	B	44	50	51	197	175	216
	C	46	38	43	416	448	429
	D	22	21	25	254	249	287
	Mean	33	34	33	252	239	249
20	A	20	16	17	78	51	72
	B	52	56	45	175	151	157
	C	40	46	44	471	535	532
	D	28	31	27	366	307	283
	Mean	35	37	33	273	261	261

eosinophils as a result of the injection of histamine and the mean counts remained approximately the same before, during and after the injections. The striking fall and subsequent rise in the numbers of these cells after a single dose of cortisone or corticotrophin are shown in Fig. 2. No increase in the amount of free histamine in the urine occurred during the period of action of either hormone.

When all the values for free histamine were plotted against the urine volume of the corresponding two-hour sample, there was no evidence of a relationship between the amount of free histamine excreted and the urine volume, thus confirming the findings of previous authors (Anrep *et al.*, 1944).

DISCUSSION

During starvation free histamine is excreted at a relatively constant rate, so that there is little variation in the amounts excreted during consecutive periods of two hours (Mitchell and Code, 1954a). The preliminary experiment was designed to determine the shortest period of fasting necessary to eliminate digestive free histamine from the urine, and the result indicates that a period of ten hours is sufficient. Urine was collected at intervals of two hours because more frequent collection may result in failure to obtain the minimum amount (50 ml.) required for the determination of free histamine. The histamine was injected in divided doses so that the total amount given during the period of two hours could be as large as possible. Little discomfort was felt during the first two experiments of each series, but headache and flushing were experienced when the total dose of histamine acid phosphate was 20 μg./kg.

These experiments confirm Adam's report (1950) that histamine administered parenterally to human beings is excreted in the free form in the urine, and indicate that the amount appearing in the urine is proportional to the dose administered. The results suggest that, as the dose of histamine injected subcutaneously is increased, the percentage excreted in the urine may also increase. However, the extra histamine excreted when 5 μg. of histamine acid phosphate/kg. was injected was so close to the fasting level of excretion that the possibility of error is greater than when 10 or 20 μg./kg. was injected, and the difference between the mean percentages excreted at the latter dosage levels is not significant ($P>0.3$). If the results of all 12 experiments are considered together, the mean percentage of the dose excreted is 0.96, a value similar to that of 1.15% found by Adam and his colleagues (1954) during the intravenous infusion of histamine, in doses comparable with those used in the present work. It seems probable therefore that within these dose ranges about 1% of the injected dose appears in the urine, presumably because these amounts are well below the saturation level of the enzyme systems destroying histamine.

There is some evidence that histamine stimulates the adrenal cortex by the release of corticotrophin (Nasmyth, 1951 ; Gray and Munson, 1951). The administration of cortisone to human beings over a period of days causes a slight increase in urinary free histamine (Mitchell and Code, 1954b); it might be argued, therefore, that the extra free histamine in the urine was not that originally injected, but was liberated as a result of pituitary-adrenal stimulation. However, cortisone also causes a fall in the numbers of basiphils and eosinophils in the blood (Code, Mitchell, and Kennedy, 1954) and no such fall occurred during the present experiments. Moreover, no increase in urinary free histamine followed the administration of a single dose of either cortisone or corticotrophin, although in each case a fall in the numbers of basiphils and eosinophils occurred (Fig. 2). It does not therefore appear likely that these hormones play any part in histamine excretion following its subcutaneous injection.

Histamine injected into the body is largely destroyed by enzymes, the small amount appearing free in the urine probably representing that fraction which escapes destruction. The close correspondence between the proportions of the

dose excreted after subcutaneous and intravenous injections of histamine suggests that all, or nearly all, of the histamine injected subcutaneously reaches the blood stream unchanged, and that little is destroyed at the site of injection.

Histamine in human blood is normally carried in the basiphils and also, though less constantly, in the eosinophils (Code and Mitchell, 1957); very little histamine is present in human plasma (Lowry, Graham, Harris, Priebet, Marks, and Bregman, 1954). If the histamine were conveyed from the site of injection to the kidneys by the circulating basiphils and eosinophils, an increase in the numbers of these cells might have been expected during the experiments; the absence of significant change suggests that the histamine was conveyed in the plasma, possibly in a loosely bound form (Emmelin, 1945). Although Adam (1950) found no rise in the plasma histamine concentration during the intravenous infusion of histamine, he and his colleagues (1954) did find indirect evidence of such an increase by the effect of the infusion on the secretion of gastric juice.

The experimental routine described in this paper has been used as a histamine excretion test in clinical studies of histamine metabolism. When the total dose of histamine acid phosphate used is 10 μg./kg. no discomfort is caused to the patient, and the test is easily carried out under clinical conditions.

Summary

1. When histamine acid phosphate was injected subcutaneously in healthy men in doses from 5 to 20 μg./kg. of body weight over 2 hr., about 1% of the dose was excreted as free histamine during the same 2-hr. period.

2. The extra free histamine excreted is thought to be that portion of the injected histamine which escaped enzymic destruction. It did not appear as the result of pituitary-adrenal stimulation or the trauma of the injection.

3. The histamine was probably not conveyed from the site of injection to the kidneys by the basiphil or eosinophil leucocytes, since the numbers of these cells in the blood did not alter significantly as a result of the histamine injections.

4. The excretion test described is readily applicable to the investigation of histamine metabolism under clinical conditions.

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Brit. J. Pharmacol. (1956), **11**, 467.

THE EFFECT OF ENZYME INHIBITORS ON THE EXCRETION OF FREE HISTAMINE IN HUMAN URINE

BY

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There is abundant evidence that most of the histamine entering or formed in the body tissues is destroyed by enzymic action (Tabor, 1954; Schayer, 1956). The enzyme mainly responsible for its destruction has been called histaminase (Best, 1929) and diamine oxidase (Zeller, 1938), but recently Schayer, Kennedy, and Smiley (1953) have demonstrated that more than one enzyme system may be involved. Using radioactive histamine and various enzyme-inhibiting drugs, these workers found that the enzyme system corresponding to diamine oxidase could be inhibited *in vivo* by aminoguanidine and by isoniazid. This enzyme system is important in the rat, but in the mouse they found that another enzyme, inhibited by iproniazid, is more important; they called this enzyme histamine-metabolizing enzyme II. Schayer and his colleagues did not extend their observations to human beings, and the present work was undertaken to investigate the effect of the enzyme-inhibiting drugs in man and, if possible, to determine the relative importance of the two enzymes in the human body. Since ^{14}C-labelled histamine cannot be used in experiments on man, the drugs were tested by assessing their effects on the excretion of free histamine in the urine after the injection of non-isotopic histamine.

Preliminary Observations on Toxicity of Enzyme Inhibitors

Aminoguanidine, in the form of aminoguanidine bicarbonate, is a tasteless white powder almost insoluble in water. Since no report could be found of its administration to human beings, tests of its toxicity to animals were first carried out. When given as an aqueous suspension to albino mice, aminoguanidine bicarbonate had an acute oral LD50 of 3 to 4 mg./g., whereas a solution of aminoguanidine chloride had an LD50 of 2 mg./g. A dose of 0.05 mg./g. of aminoguanidine bicarbonate was given daily by mouth to 20 mice for two periods of five days, separated by an interval of two days. No deaths occurred and no toxic signs were observed. A solution of aminoguanidine chloride injected intravenously into a cat under chloralose anaesthesia had little effect on the blood pressure until doses of 20 to 100 mg./kg. were reached, when a brief fall in blood pressure (5 to 10 mm. Hg) occurred, followed by a rise of 10 to 15 mm. Hg lasting 2 to 3 min., and accompanied by a slight reduction in cardiac and respiratory rates. Even doses up to 100 mg./kg. had no effect on the depressor response to 1 μg. of histamine.

Aminoguanidine was given by mouth to two groups of three guinea-pigs, in doses of 200 mg./kg. daily for 7 days to the first group, and 1,000 mg./kg. on a single occasion to the second. There was no significant effect on the haemoglobin level, red cell count or total and differential white cell counts, estimated before, during, and at seven days after administration of the compound.

Isoniazid and iproniazid were given to human subjects by mouth in powder form as "Rimifon" (Roche Products) and "Marsilid" (Roche Products) respectively. A relatively small dose was given at first, and increased in successive experiments. When 25 mg. isoniazid/kg. was given, vomiting and slight haematemesis occurred one hour later, and it was therefore considered inadvisable to give a larger dose. The vomiting was probably not a central effect, however, but rather a sign of gastric intolerance occasioned by the conditions of the experiment (the subject was fasting), since Schmidt, Hoffmann, and Hughes (1953) found that monkeys could tolerate 40 mg. isoniazid/kg. in a single dose and there is evidence that human tolerance is similar to that of monkeys (Sullivan, Barclay, and Karnofsky, 1954).

No toxic effects were noted when iproniazid was given in doses up to 25 mg./kg., but since it is generally regarded as the more toxic drug in man (Selikoff, Robitzek, and Ornstein, 1952), the maximum dose of this drug was also limited to 25 mg./kg.

METHODS

The methods used and the general plan of the experiments have been described (Mitchell, 1956). Free histamine was extracted from urine by the method of Roberts and Adam (1950) and was assayed on a strip of guinea-pig ileum suspended in atropinized Tyrode solution. All values for histamine are given in terms of histamine base, unless stated otherwise.

Tests were carried out to make sure that small amounts of the drugs excreted in the urine did not

interfere with the extraction or assay of histamine. When aminoguanidine bicarbonate, isoniazid or iproniazid was added to urine before extraction to give a concentration of 50 mg. or 100 mg./50 ml., there was no significant difference between estimates of the free histamine content of the original urine and of the duplicate sample to which the drug had been added.

The experiments were carried out on healthy men between 25 and 35 years of age. At least four weeks elapsed between experiments on any individual. In each experiment, urine was collected at intervals of 2 hr. over a period of 8 hr. (from 5.30 a.m. to 1.30 p.m.) and the amount of free histamine determined in each of the four samples. The subject fasted for 10 hr. before the test and throughout its duration, but was allowed to drink water.

In the first series of experiments, aminoguanidine was given to each of three subjects (X, Y, and Z) on two occasions, in a dose of 10 mg./kg. of body weight in the first test, and 20 mg./kg. in the second. The drug was given by mouth half an hour before the end of the second collection period (at 9 a.m.).

The next series of experiments was carried out on the same three men (subjects X, Y, and Z). A total of 10 μg. of histamine acid phosphate/kg. was injected subcutaneously during the third collection period, in four equal doses at intervals of half an hour, starting at 9.30 a.m. This "histamine excretion test" was performed three times on each subject. In the first test no additional drug was given, while in the second and third aminoguanidine was given at 9 a.m. in doses of 5 mg. and 10 mg./kg. respectively.

In the final series of experiments the histamine excretion test was carried out as before, seven tests being made on one subject. Isoniazid was given by mouth at 9 a.m., in a dose of 5 mg./kg. in the first test, 10 mg./kg. in the second, and 25 mg./kg. in the third. In the next four experiments, iproniazid was given in the same way in doses of 5, 10, 15, and 25 mg./kg.

In all the experiments, extraction of free histamine was started within an hour of collection of the last specimen of urine.

Results

The amounts of free histamine excreted during the first and second two-hour periods in all the experiments ranged from 0.28 μg. to 1.20 μg., a range similar to that of 0.37 μg. to 1.10 μg. found in other healthy men (Mitchell, 1956). These values represent the basal excretion of free histamine when the subject is fasting.

The results of the experiments with aminoguanidine are shown in Table I. In the first six tests, when aminoguanidine was given without subsequent injection of histamine, the mean values indicate a small increase in the excretion of histamine in periods III and IV. This increase was

TABLE I

THE EFFECT OF AMINOGUANIDINE ON THE EXCRETION OF METABOLIC FREE HISTAMINE AND ON THE EXCRETION OF SUBCUTANEOUSLY INJECTED HISTAMINE IN THE URINE OF THREE HEALTHY MEN

Oral Dose of Aminoguanidine Bicarbonate Given at 9 a.m. (mg./kg.)	Total Dose of Histamine Acid Phosphate Injected during Period III (μg./kg.)	Subject and Body Weight (kg.)	Free Histamine in Urine in μg. Histamine Base/2-hr. Period				Extra Histamine Excreted in Period III as % of Total Dose Injected
			I 5.30–7.30	II 7.30–9.30	III 9.30–11.30	IV 11.30–1.30	
10	Nil	X. 75	0·46	0·51	0·88	0·72	—
		Y. 66	0·43	0·40	0·62	1·06	—
		Z. 72	0·39	0·42	0·35	0·80	—
		Mean	0·43	0·44	0·62	0·86	—
20	"	X. 75	0·74	0·72	1·61	1·33	—
		Y. 66	—*	0·72	1·82	0·93	—
		Z. 72	0·66	0·74	0·94	1·00	—
		Mean	0·70	0·73	1·46	1·09	—
Nil	10	X. 75	0·54	0·59	2·59	0·63	1·18
		Y. 66	0·80	0·83	1·87	0·76	0·70
		Z. 72	0·38	0·42	1·77	0·70	0·83
		Mean	0·57	0·61	2·08	0·70	0·90
5	10	X. 75	0·35	0·40	4·66	0·93	2·46
		Y. 66	0·85	0·60	3·80	0·70	2·03
		Z. 72	0·60	0·87	4·28	0·98	2·15
		Mean	0·60	0·62	4·25	0·87	2·21
10	10	X. 75	0·55	0·73	4·86	1·43	2·45
		Y. 66	—*	1·20	6·14	0·90	3·27
		Z. 72	0·37	0·36	6·00	0·63	3·42
		Mean	0·46	0·76	5·67	0·99	3·05

* Volume of sample inadequate.

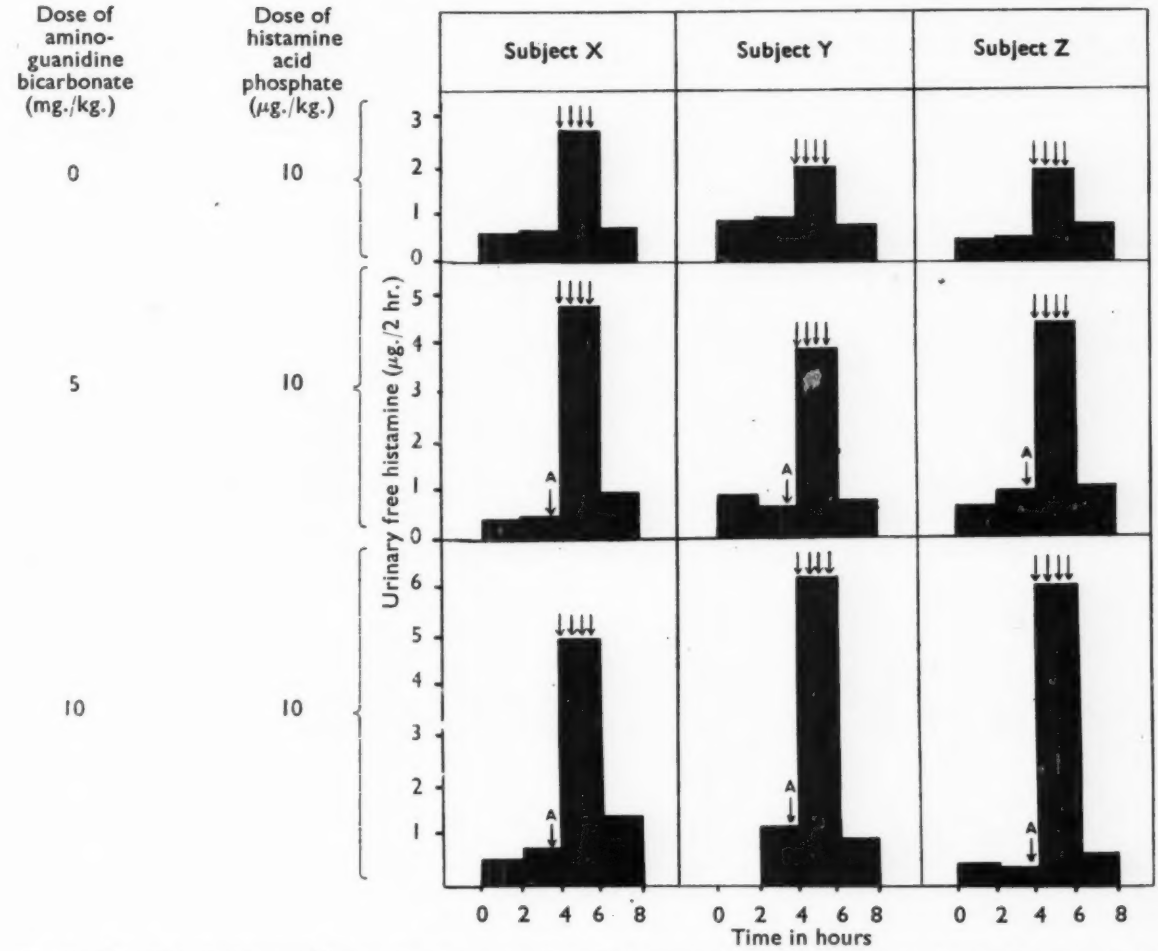

FIG. 1.—The effect of aminoguanidine on the urinary excretion of subcutaneously injected histamine by three healthy men. Total dose of histamine acid phosphate injected in four doses as indicated by the sets of 4 arrows. Aminoguanidine given in one oral dose at arrows marked A.

barely significant, but in all six experiments the rate of excretion in period IV was higher than in either control period in the same test. Such consistency can be considered significant.

The effect of aminoguanidine on the excretion of free histamine was more definite when histamine was injected subsequently (Table I and Fig. 1). When the histamine excretion test was carried out without giving aminoguanidine, the mean value for the extra free histamine excreted during period III was 1.49 μg., representing 0.90% of the dose administered. This mean rate is not significantly different ($P=0.35$) from the mean rate of 1.12% recorded previously for this test (Mitchell, 1956), the calculations being made as described in that paper. If the results of these two series of tests are combined, the mean percentage of the dose excreted for the seven tests is 1.02% (range 0.70% to 1.32%).

When aminoguanidine was given in a dose of 5 mg./kg. half an hour before the first injection of histamine, the mean value for the extra free histamine excreted in period III represented 2.21% of the dose. This mean rate of excretion is significantly higher ($P<0.01$) than the mean rate when no aminoguanidine was given. Aminoguanidine in a dose of 10 mg./kg. resulted in an even greater excretion of free histamine in period III, the mean value representing 3.05% of the dose administered.

When isoniazid or iproniazid in doses up to 25 mg./kg. were administered (Table II) there was no definite effect on the histamine excretion test.

DISCUSSION

Following the parenteral injection of histamine, there is an increase in urinary free histamine amounting to about 1% of the dose administered (Adam, 1950; Mitchell, 1956). Most of the

TABLE II

THE EFFECTS OF ISONIAZID AND IPRONIAZID ON THE EXCRETION OF FREE HISTAMINE IN THE URINE OF A HEALTHY MAN (BODY WEIGHT 80 KG.) AFTER THE INJECTION OF 10 μG. HISTAMINE ACID PHOSPHATE/KG. OVER A PERIOD OF 2 HR. (PERIOD III)

Drug Given as a Single Oral Dose at 9 a.m.	Dose (mg./kg.)	Free Histamine in Urine in μg. Histamine Base/2-hr. Period				Extra Histamine Excreted in Period III as % of Total Dose Injected
		I 5.30–7.30	II 7.30–9.30	III 9.30–11.30	IV 11.30–1.30	
Isoniazid (Rimifon, Roche)	5	0·40	0·45	2·46	0·63	1·11
	10	0·39	0·38	2·63	0·56	1·23
	25	0·74	0·59	3·29	0·67	1·43
Iproniazid (Marsilid, Roche)	5	0·45	0·52	2·83	0·57	1·28
	10	0·66	0·64	3·05	0·85	1·31
	15	0·35	0·41	2·38	0·90	1·09
	25	0·28	0·60	2·85	0·58	1·31

remaining 99% is thought to be destroyed by enzymes in the body (Gaddum, 1951). Previous work suggested that histamine injected subcutaneously is not destroyed at the site of injection (Mitchell, 1956) but reaches the blood stream unchanged. The fact that aminoguanidine injected intravenously had no effect on the depressor response to histamine is consistent with the findings for other histaminase inhibitors (Arunlakshana, Mongar, and Schild, 1954), and suggests that, in the cat at least, little or no enzymic destruction of exogenous histamine takes place in the plasma. This probably also applies to man, whose pattern of histaminase distribution is similar to that of the cat (Kahlson, 1956). Subcutaneously injected histamine may therefore be expected to reach the general body tissues virtually unchanged, since little is likely to be destroyed in the lungs, which in man contain very little histaminase (Zeller, Birkhauser, Mislin, and Wenk, 1939). Most of the histamine reaching the kidney will be destroyed by the rich stores of histaminase in that organ (Waton, 1956), only a fraction being excreted as free histamine. It is apparent therefore that even slight inhibition of the enzymes concerned may be expected to produce a considerable increase in urinary free histamine.

The most suitable time to give the enzyme-inhibiting drug was estimated from the known facts about isoniazid. Absorption of this drug from the stomach is rapid and complete, the peak plasma level being reached between half an hour and two hours after administration (Elmendorf, Cawthon, Muschenheim, and McDermott, 1952; Rubin and Burke, 1953). The drug was therefore given half an hour before the first injection of histamine, so that the peak plasma level would be reached some time between the first and last injections. The same observations apply to iproniazid, which also reaches peak plasma levels very rapidly after oral administration (Rubin, Drekter, Scheiner, and de Ritter, 1952).

Since nothing is known of the absorption of aminoguanidine in man, this compound was also given half an hour before the first histamine injection. It is apparent from the results in Table I that some of the drug must have been absorbed rapidly, although the results of the first six tests indicate that a slightly longer interval between the administration of the drug and the first histamine injection might have produced a greater effect. Furthermore, the results of the tests on albino mice suggest that a solution of aminoguanidine chloride might have been more rapidly and completely absorbed than the almost insoluble bicarbonate.

The effect of aminoguanidine on the excretion of injected histamine is presumably due to inhibition of histaminase in the kidney, a view supported by the experimental work of Lindell and Westling (1956) in cats. In rats a definite increase in urinary histamine occurs after the subcutaneous injection of only 0.5 mg. aminoguanidine chloride/kg. whereas 10 mg./kg. almost stops diamine oxidase (histaminase) activity (Schayer, Kennedy, and Smiley, 1953). Even allowing for the lesser efficacy of the bicarbonate and the possibility of incomplete absorption from the stomach, the comparatively small response to oral doses of 5 and 10 mg. aminoguanidine bicarbonate/kg. suggests that, in the human body, histaminase is not so readily inhibited by aminoguanidine as in the rat. Nevertheless, the results confirm that this enzyme is important in the destruction of histamine in the human body.

Schayer (1953) reported that, in rats, 10 mg. isoniazid/kg. had a slight effect on histamine excretion and 50 mg./kg. had a pronounced effect. Since isoniazid is as effective orally as parenterally, the dose of 25 mg./kg. in the human subject might have been expected to produce a definite increase in the excretion of free histamine. That it did not do so simply confirms that hista-

minase in the human body is less readily inhibited than in the rat.

Iproniazid inhibits histamine-metabolizing enzyme II, which is thought to be a composite of a methylating enzyme and monoamine oxidase (Schayer, 1956). Iproniazid in a dose of 190 to 200 mg./kg. had a pronounced inhibitory effect on monoamine oxidase in mice (Schayer, 1953) and in rats (Schayer and Smiley, 1953), but a dose of 100 mg./kg. had a slight effect. Zeller and Barsky (1952) reported inhibition of monoamine oxidase activity in rats with as little as 28 mg./kg. It appears probable, therefore, that histamine-metabolizing enzyme II is not of major importance in man, since iproniazid in doses up to 25 mg./kg. had no effect at all on histamine excretion.

The results of the animal tests confirm Alles' report (1926) that aminoguanidine is relatively non-toxic, and no toxic effects of the drug were observed in the human subjects. Aminoguanidine may, however, cause changes in the blood like those of pernicious anaemia (Lieber and Smith, 1939) and such a possibility would have to be considered if the drug were given over a period. The histamine excretion test caused no significant symptoms, but when aminoguanidine was given before the injections, headache and flushing occurred. These were, of course, histamine effects and not attributable directly to the aminoguanidine.

The low toxicity of aminoguanidine and its effect on histamine metabolism indicate that it might be of therapeutic value in any condition in which histaminase activity is excessive. In pregnancy the plasma contains an abnormally large amount of histaminase, but this may be necessary for the maintenance of normal pregnancy, and the administration of aminoguanidine is dangerous, since it may interrupt the course of pregnancy (Roberts, 1954). Apart from pregnancy, no other condition has so far been recognized in human beings in which there is excessive production of histaminase (Kapeller-Adler, 1956).

Summary

1. The oral administration of aminoguanidine to healthy men in doses of 10 to 20 mg./kg. of body weight resulted in a slight increase in the excretion of free histamine in the urine.

2. When histamine is injected subcutaneously, 1% of the dose normally appears in the urine in the free form. This rate of excretion increased to about 2% when 5 mg. of aminoguanidine/kg. was given by mouth before the injection of histamine and to about 3% when 10 mg./kg. was given. The increase is attributed to inhibition of histaminase (diamine oxidase) by the aminoguanidine.

3. No significant increase in the rate of excretion of subcutaneously injected histamine followed the oral administration of isoniazid (Rimifon) or iproniazid (Marsilid) in doses up to 25 mg./kg.

4. The results are interpreted as indicating that histaminase is the principal enzyme destroying histamine in the human body.

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THE METABOLISM OF HISTAMINE IN VARIOUS SPECIES

BY

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This laboratory has demonstrated that in all mammalian species tested—rats (Schayer, 1953), mice, guinea-pigs (Schayer and Karjala, 1956), and man (Schayer and Cooper, 1956)—there are two major histamine-metabolizing enzymes. One is diamine oxidase; it leads to the production of imidazole-4-acetic acid and l-ribosylimidazole-4(5)-acetic acid (Karjala, Turnquest, and Schayer, 1956). The second enzyme methylates histamine on the ring nitrogen remote from the side chain. It leads to the formation of l-methyl-4-(β-aminoethyl)imidazole and l-methylimadazole-4-acetic acid. These four metabolites will be referred to as ImAA, ImAA-riboside, 1,4-methylhistamine, and 1,4-methyl ImAA respectively.

In this paper are reported quantitative analyses for histamine metabolites in the urine of various species after feeding or injecting ^{14}C histamine. An experiment to identify the precursor of bound histamine in the cat is also reported.

TABLE I

QUANTITATIVE ANALYSIS FOR HISTAMINE METABOLITES IN THE URINE OF VARIOUS SPECIES AFTER FEEDING OR INJECTING ^{14}C HISTAMINE

	Histamine Metabolites, % of Total ^{14}C in Urine					
	Histamine	1,4-Methyl Histamine	1,4-Methyl ImAA	ImAA Free	ImAA Riboside	Acetylhistamine
Rabbits, 2.0 and 2.1 kg. 60 μg. ^{14}C histamine each, subcutaneously, combined urine† ..	—*	—	41	Trace	40	0·5
Cat, 2·5 kg. male, 50 μg. ^{14}C histamine subcutaneously ..	9	—	72	—	—	0
Cat, 0·6 kg. female, fed 100 μg. ^{14}C histamine	0·3	0·6	77	5	4	—
Mice, four, average wt. 21 g. female, each fed 14 μg. ^{14}C histamine	—	—	8	18	47	—
Man, 85 kg. male, Subject H.K. fed 100 μg. ^{14}C histamine ..	0	—	64	—	—	—
Dog, 22 kg. mongrel, male, 120 μg. ^{14}C histamine subcutaneously	0	7	61	21	0 to 1‡	—
Dog, 9 kg. mongrel, male, 60 μg. ^{14}C histamine subcutaneously	0	9	66	—	—	—

* A line indicates that the assay was not performed.

† Most urine samples were collected for a period of 6 hours following the administration of ^{14}C histamine; the only exception was the 22 kg. dog whose urine was collected for 27 hours.

‡ The value obtained for total ImAA was 22%. Since the value obtained for free ImAA was 21% there is no evidence for the presence of conjugated ImAA.

METHODS

The determination of urinary metabolites of histamine by isotope dilution has been described (Schayer and Karjala, 1956; Schayer and Cooper, 1956). ^{14}C histamine in cat tissues was determined by addition of carrier followed by the usual extraction procedure (Schayer, 1952). The carrier histamine was converted to the dibenzenesulphonyl derivative (Schayer, 1956) and counted in a Packard Tri-Carb Liquid Scintillation Spectrometer (background 10–11 counts/min.).

RESULTS

Various species were given ^{14}C histamine by feeding or by subcutaneous injection. Urine was collected in vessels containing hydrochloric acid and toluene. Complete analysis for all known metabolites were not done on all samples; in some cases only those assays were performed which were required to show the major pathway of histamine metabolism. The experimental conditions and results of assays are summarized in Table I.

DISCUSSION

From the data of Table I the following inferences can be made:

In the rabbit histamine is metabolized by oxidation and by methylation to approximately the same extent.

The mouse, which methylates most injected histamine (Schayer and Karjala, 1956), metabolizes most intestinal histamine by oxidation, not by methylation.

In cats and man,* on the contrary, methylation is the principal route of metabolism for both fed

* The percentage of total ingested ^{14}C in the urine of the human subject H. K. at various time intervals was: 0-6 hr., 40%; 6-12 hr., 14%; 12-24 hr., 7%; 24-48 hr., 5%. Total recovery in 48 hr. was 66%.

and injected histamine. In man, no fed histamine could be detected unchanged in the urine.

In the dog, methylation is the main means of inactivation of injected histamine. Dogs are the only animals tested in which none of the injected histamine could be detected unchanged in the urine. Furthermore, dogs do not appear to conjugate appreciable quantities of ImAA with ribose.

To test the recent suggestion by Waton (1956) that intestinal histamine might be a source of bound histamine in carnivores, the cat which had been fed ^{14}C histamine was killed 8 days after the feeding and various tissues assayed for ^{14}C histamine content. This cat had received 4.6 microcuries of radioactive histamine. Results in counts per minute were: intestine (60 g.) 2, skin (70 g.) 1, stomach (64 g.) 2, and lung (64 g.) 3. These counts are too low to indicate the presence of ^{14}C histamine.

On the other hand, another cat (a litter mate of the first cat) 8 days after injection with ^{14}C L-histidine had definitely detectable quantities of ^{14}C histamine in its tissues. The values in counts per minute were: intestine (sample lost), skin (48 g.) 73, stomach (39 g.) 71, and lung (46 g.) 29. This cat received 20 microcuries of ^{14}C L-histidine; however, it should be emphasized that only a minute fraction of injected L-histidine is converted to histamine. These results suggest that in cats, as in other species (Schayer, 1952; Schayer and Smiley, 1954), the precursor of bound histamine is L-histidine and not exogenous histamine.

Summary

Quantitative analyses for histamine metabolites in the urine of various species are reported. An experiment is described which identifies L-histidine as the precursor of bound histamine in the cat.

This work was supported by a contract with the United States Atomic Energy Commission. The author is indebted to Rosa L. Smiley for technical assistance. The ^{14}C histamine was fed to subject H. K. by Dr. John A. D. Cooper, of the Northwestern University Medical School; permission for this experiment was granted by the U.S. Atomic Energy Commission.

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INDEX TO VOLUME 11

AUTHOR INDEX

A

ABRAHAMS, V. C., and PICKFORD, M. Effect of 5-hydroxytryptamine on excretion of water in conscious dogs, 35

——— ———. Effect of 5-hydroxytryptamine on ureter and on blood pressure of dogs; and of adrenaline, noradrenaline, and posterior pituitary extracts on ureter, 44

——— ———. Effect of 5-hydroxytryptamine on uterus of conscious and of anaesthetized dogs, 50

AMBACHE, N., PERRY, W. L. M., and ROBERTSON, P. A. Effect of muscarine on perfused superior cervical ganglia of cats, 442

ANGELUCCI, L. Experiments with perfused frog's spinal cord, 161

ASKEW, B. M. Oximes and hydroxamic acids as antidotes in anticholinesterase poisoning, 417

———, DAVIES, D. R., GREEN, A. L., and HOLMES, R. Nature of the toxicity of 2-oxo-oximes, 424

B

BAIN, W. A., and BATTY, J. E. Inactivation of adrenaline and noradrenaline by human and other mammalian liver *in vitro*, 52

BARLOW, R. B., and VANE, J. R. Ganglion-blocking properties of hexamethylenebisdialkylsulphonium salts, 198

BATTY, J. E. *See* BAIN, W. A., 52

BELFORD, J. *See* KAO, F. F., 15

BHATTACHARYA, B. K., and LEWIS, G. P. Effects of reserpine and Compound 48/80 on the release of amines from mast cells of rats, 411

——— ———. Release of 5-hydroxytryptamine by histamine liberators, 202

BIANCHI, C. Anticonvulsant action of some anti-epileptic drugs in mice pre-treated with *Rauwolfia* alkaloids, 141

———. Simple new quantitative method for testing local anaesthetics, 104

BLACKMAN, J. G., FASTIER, F. N., PATEL, C. M., and WONG, L. C. K. Assessment of depressor activity and mydriatic activity of hexamethonium analogues, 282

BROWN, B. G., and HEY, P. Choline phenyl ethers as inhibitors of amine oxidase, 58

BROWN, L. M. *See* GINSBURG, M., 236

BRZIN, M., and ŽUPANČIČ, A. O. Mode of action of tubocurarine, 428

BURGEN, A. S. V., BURKE, G., and DESBARATS-SCHONBAUM, M. Specificity of brain choline acetylase, 308

BURKE, G. *See* BURGEN, A. S. V., 308

C

CALDWELL, A. G., and STANDEN, O. D. Activity of *p*-aminophenoxyalkane derivatives against *Schistosoma mansoni*, 367

CATER, D. B. *See* PHILLIPS, A. F., 128

CHESHER, G. B. *See* COLLIER, H. O. J., 20, 180

COCHRAN, J. B., and RAMSAY, A. G. Hyperpnoea produced by intravenous administration of salicylates, 364

COLES, D. R., DUFF, F., SHEPHERD, W. H. T., and WHELAN, R. F. Effect on respiration of infusions of adrenaline and noradrenaline into the carotid and vertebral arteries in man, 346

COLLIER, H. O. J., and CHESHER, G. B. Antipyretic and analgesic properties of two hydroxy*iso*phthalic acids, 20

——— ———. Identification of 5-hydroxytryptamine in the sting of the nettle (*Urtica dioica*), 186

CONWAY, J. Role of blood-pressure level in production of ventricular arrhythmia in the rabbit, 132

D

DAVIES, D. R. *See* ASKEW, B. M., 424

DAVIES, G. E., DRIVER, G. W., HOGGARTH, E., MARTIN, A. R., PAIGE, M. F. C., ROSE, F. L., and WILSON, B. R. Chemotherapy of tuberculosis: Ethyl mercaptan and related compounds, 351

DESBARATS-SCHONBAUM, M. *See* BURGEN, A. S. V., 308

DIDCOCK, K. A., JACKSON, D., and ROBSON, J. M. Action of some nucleotoxic substances on pregnancy, 437

———. *See also* ROBSON, J. M., 190

DRIVER, G. W. *See* DAVIES, G. E., 351

DUFF, F. *See* COLES, D. R., 346

DUFF, R. S., and GINSBURG, J. Antagonism between chlorpromazine and noradrenaline in blood vessels of the hands, 318

———. *See also* GINSBURG, J., 180

DUNDEE, J. W. Effect of thiopentone on blood sugar and glucose tolerance, 458

E

ELIASSON, R., LIE, L., and PERNOW, B. Comparative study of Substance P from intestine and brain, 137

F

FASTIER, F. N. *See* BLACKMAN, J. G., 282

FRASER, P. J. Acceleration of the enzymic hydrolysis of benzoylcholine, 7

FRAZER, J. F. D. Sperm-shedding response of male toads and treefrogs after injection of two types of gonadotrophin, 248

G

GABR, Y. Substance in human plasma giving slow contraction of guinea-pig gut *in vitro*, 93

GADDUM, J. H. Estimation of the safe dose, 156

——— and GIARMAN, N. J. Preliminary studies on the biosynthesis of 5-hydroxytryptamine, 88

——— and VOGT, M. Some central actions of 5-hydroxytryptamine and various antagonists, 175

GARVEN, J. D. Estimation of 5-hydroxytryptamine in the presence of adrenaline, 66

GERTNER, S. B. Pharmacological studies on the inferior eyelid of anaesthetized rat, 147
GIARMAN, N. J. *See* GADDUM, J. H., 88
GINSBURG, J., and DUFF, R. S. Effect of chlorpromazine on adrenaline vasoconstriction in man, 180
———. *See also* DUFF, R. S., 318
GINSBURG, M. Antidiuretic activity of posterior pituitary preparations, 245
——— and BROWN, L. M. Effect of anaesthetics and haemorrhage on release of neurohypophysial antidiuretic hormone, 236
GODFREY, D. G. *See* TAYLOR, A. E. R., 71
GOODALL, R. R. Mepacrine metabolism: examination of mouse liver for possible antiviral metabolites, 215
GRAHAM, J. D. P., and TONKS, R. S. Series of fully substituted ethylenediamines, 1
GREEN, A. F., and WARD, N. B. Analgesic and other properties of morpholinoethylnorpethidine, 32
GREEN, A. L. *See* ASKEW, B. M., 424
GREENHALGH, N., HULL, R., and WESTON HURST, E. Antiviral activity of acridines in eastern equine encephalomyelitis, Rift Valley fever and psittacosis in mice, and lymphogranuloma venereum in chick-embryos, 220
GRUNDY, H. F., and HOWARTH, F. Pharmacological studies on hemlock water dropwort, 225

H

HAINING, C. G. Activation of rabbit serum protease by dextran sulphate, 107
———. Inhibition of histamine release by sodium salicylate and other compounds, 357
HENRIQUES, O. B. *See* HENRIQUES, S. B., 99
HENRIQUES, S. B., HENRIQUES, O. B., and LEVY, A. Slow hyperfibrinogenaemic action of adrenaline and related substances, 99
HEY, P. *See* BROWN, B. G., 58
HOBBIGER, F. Chemical reactivation of phosphorylated human and bovine true cholinesterases, 295
HOGGARTH, E. *See* DAVIES, G. E., 351
HOLLAND, W. C., and SCHÜMANN, H. J. Formation of catechol amines during splanchnic stimulation of the adrenal gland of the cat, 449
HOLMES, R. *See* ASKEW, B. M., 424
HOWARTH, F. *See* GRUNDY, H. F., 225
HULL, R. *See* GREENHALGH, N., 220

J

JACKSON, D. *See* DIDCOCK, K. A., 437
JOYNER, L. P., and KENDALL, S. B. Mode of action of a mixture of pyrimethamine and sulphadimidine on *Eimeria tenella*, 454

K

KAO, F. F., and BELFORD, J. Analysis of the central respiratory action of nalorphine in decerebrate dogs, 15
KAY, A. W., and SMITH, A. N. Action of atropine and hexamethonium in combination on gastric secretion and motility, 231
KENDALL, S. B. *See* JOYNER, L. P., 454
KONZETT, H. Effects of 5-hydroxytryptamine and its antagonists on tidal air, 289

L

LEVY, A. *See* HENRIQUES, S. B., 99
LEWIS, G. P. *See* BHATTACHARYA, B. K., 202, 411
LIE, L. *See* ELIASSON, R., 137

M

MARSON, F. G. W. Effect of noradrenaline on urine and renal blood flow, 431
MARTIN, A. R. *See* DAVIES, G. E., 351
MILLAR, R. A., and STEPHENSON, R. P. Analgesic action in a series of *N*-substituted ethyl 4-phenylpiperidine-4-carboxylates, 27
MITCHELL, R. G. Effect of enzyme inhibitors on excretion of free histamine in human urine, 467
———. Urinary excretion of subcutaneously injected histamine, 462
MOTA, I., and VUGMAN, I. Action of Compound 48/80 on mast cells and histamine content of guinea-pig tissues, 304

N

NAGUIB, M., and ROBSON, J. M. Effect of cortisone alone and in combination with isoniazid on experimental murine leprosy in mice, 326
NICHOLLS, J. G., and QUILLIAM, J. P. Mechanism of action of paraldehyde and methylpentynol on neuromuscular transmission in frog, 151

O

ORMEROD, W. E. Pharmacology of benzoylcholine derivatives and nature of carbonyl receptors, 267
ÖSTLUND, E. *See* VON EULER, U. S., 323

P

PAIGE, M. F. C. *See* DAVIES, G. E., 351
PATEL, C. M. *See* BLACKMAN, J. G., 282
PERNOW, B. *See* ELIASSON, R., 137
PERRY, W. L. M., and WILSON, C. W. M. Relative effects of ganglion-blocking compounds on the sympathetic and parasympathetic ganglia supplying cat heart, 81
———. *See also* AMBACHE, N., 442
PHILLIPS, A. F., and CATER, D. B. Protection by cysteine against the acute toxicity of a chemical radiosensitizer (" Synkavit "), 128
PICKFORD, M. *See* ABRAHAMS, V. C., 35, 44, 50
POLLER, L. Effect of acetazoleamide, an inhibitor of carbonic anhydrase, on gastric secretion, 263

Q

QUILLIAM, J. P. *See* NICHOLLS, J. G., 151

R

RAMSAY, A. G. *See* COCHRAN, J. B., 364
RAVENTÓS, J. Action of fluothane—a new volatile anaesthetic, 394
ROBERTSON, P. A. *See* AMBACHE, N., 442
ROBSON, J. M., and DIDCOCK, K. A. Effect of isoniazid in experimental corneal tuberculosis, 190
———. *See also* DIDCOCK, K. A., 437
———. *See also* NAGUIB, M., 326
ROCHA E SILVA, M., and ROTHSCHILD, A. M. Experimental design for biossay of a material inducing strong tachyphylactic effect (anaphylatoxin), 252
ROSE, F. L. *See* DAVIES, G. E., 351
ROTHSCHILD, A. M. *See* ROCHA E SILVA, M., 252
RYALL, R. W. Some actions of chlorpromazine, 339

S

SAVINI, E. C. Antagonism between 5-hydroxytryptamine and certain derivatives of lysergic acid, 313
SCHACHTER, M. Delayed slow contracting effect of serum and plasma due to the release of a substance resembling kallidin and bradykinin, 111

SCHAYER, R. W. Metabolism of histamine in various species, 472
SCHÜMANN, H. J. *See* HOLLAND, W. C., 449
SHEA, S. M. Inhibition of gastric secretion in rats by some quaternary derivatives of atropine, 171
SHEPHERD, W. H. T. *See* COLES, D. R., 346
SMITH, A. N. *See* KAY, A. W., 231
SNOW, G. A., and WESTON HURST, E. Distribution of mepacrine in organs of different animal species, and in components of liver cells, 209
STANDEN, O. D., and WALLS, L. P. Effect of ring-substituents on the activity of diphenoxyalkanes against *Schistosoma mansoni*, 375
———. *See also* CALDWELL, A. G., 367
STEPHENSON, R. P. Modification of receptor theory, 379
———. *See also* MILLAR, R. A., 27

T

TAYLOR, A. E. R., TERRY, R. J., and GODFREY, D. G. Action of some trypanocidal and antimalarial compounds on *Babesia rodhaini* (*Piroplasmidea*), 71
TERRY, R. J. *See* TAYLOR, A. E. R., 71
TIMMS, A. R. Mode of action of some non-specific acetylcholine antagonists, 273
TONKS, R. S. *See* GRAHAM, J. D. P., 1
TRENDELENBURG, U. Action of 5-hydroxytryptamine on nictitating membrane and on superior cervical ganglion of cat, 74

V

VANE, J. R. *See* BARLOW, R. B., 198
VOGT, M. *See* GADDUM, J. H., 175
VON EULER, U. S., and ÖSTLUND, E. Occurrence of a Substance P-like polypeptide in fish intestine and brain, 323
VUGMAN, I. *See* MOTA, I., 304

W

WALLS, L. P. *See* STANDEN, O. D., 375
WARD, N. B. *See* GREEN, A. F., 32
WATON, N. G. Studies on mammalian histidine decarboxylase, 119
WESTON HURST, E. *See* GREENHALGH, N., 220
———. *See also* SNOW, G. A., 209
WHELAN, R. F. *See* COLES, D. R., 346
WILSON, B. R. *See* DAVIES, G. E., 351
WILSON, C. W. M. *See* PERRY, W. L. M., 81
WONG, L. C. K. *See* BLACKMAN, J. G., 282
WOOLFE, G. Trypanocidal action of phenanthridine compounds: effect of changing the quaternary groups of known trypanocides, 330
———. Trypanocidal action of phenanthridine compounds: further 2:7-diaminophenanthridinium compounds, 334

Z

ŽUPANČIČ, A. O. *See* BRZIN, M., 428

SUBJECT INDEX

A

2-*p*-Acetamidostyryl-6-methylaminoquinoline methosulphate, action in *Babesia rodhaini* infections, 71
Acetazoleamide, effect on gastric secretion, 263
Acetylcholine, non-specific antagonists of, mode of action, 273
(+)-1-Acetyllysergic acid diethylamide, effect on tidal-air reduction by 5-hydroxytryptamine, 289
Acridines, antiviral activities in equine encephalomyelitis, Rift Valley fever, psittacosis and lymphogranuloma venereum, 220
Actinomycin C, effect on pregnancy in animals, 437
Adrenal gland, adrenaline and noradrenaline formation in, during splanchnic stimulation in cat, 449
Adrenaline, effect on respiration, 346
———, effect on ureter in dogs, 44
———, formation during splanchnic stimulation of adrenals in cat, 449
———, inactivation by liver *in vitro*, 52
———, slow hyperfibrinogenaemic action of, 99
———, vasoconstrictor action of, effect of chlorpromazine on, in man, 180
Adrenalone, slow hyperfibrinogenaemic action of, 99
Adrenochrome, hyperfibrinogenaemic action of, 99
Alkoxy(*p*-aminophenoxy)alkanes, schistosomicidal activities of, 367
10-Allyl-7-amino-9-*p*-aminophenylphenanthridinium chloride, trypanocidal activity of, 330
10-Allyl-2:7-diamino-9-*p*-chloro-, -ethanesulphonyl- and -*iso*propyl-phenylphenanthridinium salts, trypanocidal activities of, 334
10-Allyl-2:7-diamino-9-methyl-, -phenyl-, -*p*-aminophenyl-, and -*p*-nitrophenyl-phenanthridinium salts, trypanocidal activities of, 330
Amine oxidase, inactivation by choline phenyl ethers, 58
7-Amino-9-*p*-aminophenyl-10-ethyl-, -methyl-, and -propyl-phenanthridinium chloride, trypanocidal activities of, 330
p-Aminodiphenoxyalkanes, schistosomicidal activities of, 367
———, ———, effect of ring-substituents on, 375
Aminoguanidine, effect on urinary excretion of free histamine in man, 467
4-Amino-10-methylpteroylglutamic acid, effect on pregnancy in animals, 437
9-*p*-Aminostyryl-2:7-diethoxycarbonylamino-10-ethyl- and -methyl-phenanthridinium chloride, trypanocidal activities of, 334
Ammonium molybdate, reactivation of phosphorylated true cholinesterases by, 295
Amphetamine, effect on central actions of 5-hydroxytryptamine, 175
———, hyperfibrinogenaemic action of, 99
Anaesthetics, effect on release of neurohypophysial antidiuretic hormone during haemorrhage, 236
———, Fluothane as, 394
———, local, quantitative testing of, 104
Anaphylatoxin, experimental design for bioassay of, 252
Antergan. *See* *N*-Benzyl-*N'N'*-dimethyl-*N*-phenylethylenediamine
Anticholinesterase poisoning, oximes and hydroxamic acids as antidotes in, 417
Antidiuretic activity of posterior pituitary preparations, 245
Antidiuretic hormone, neurohypophysial, effect of anaesthetics and haemorrhage on release of, 236
Anti-epileptic drugs, anticonvulsant activity in mice pretreated with *Rauwolfia* alkaloids, 141
Antihistaminics, mode of action as acetylcholine antagonists, 273

Antimalarials, action in *Babesia rodhaini* infections, 71
Antimony potassium tartrate. *See* Tartar emetic
Antrycide methyl sulphate, action in *Babesia rodhaini* infections, 71
Atropine, effect on cholinesterase activity, 7
———, effect on tidal-air reduction by 5-hydroxytryptamine, 289
———, quaternary derivatives of, gastric-secretion inhibition by, 171
———, with hexamethonium, effect on gastric secretion and motility, 231
Azamethonium. *See* Pendiomide

B

B 314. *See* 2-*p*-Acetamidostyryl-6-methylaminoquinoline methosulphate
Babesia rodhaini, action of antimalarials and trypanocides on, 71
Benzoylcholine, derivatives of, pharmacological actions of, 267
———, enzymic hydrolysis of, accelerators of, 7
5-Benzoyloxygramine, effect on central actions of 5-hydroxytryptamine, 175
N-Benzyl-*N'N'*-diethyl-*N*-naphth-1- and -2-ylethylenediamine, pharmacological properties of, 1
N-Benzyl-*N'N'*-dimethyl-*N*-naphth-1- and -2-ylethylenediamine, pharmacological properties of, 1
N-Benzyl-*N'N'*-dimethyl-*N*-phenylethylenediamine, pharmacological properties of, 1
Berenil. *See* Di(*p*-amidinophenyl)triazine diaceturate
Bioassay, experimental design for, for material inducing strong tachyphylactic effect, 252
Bis-4-amino-2-methylquinolyl-6-melamine, action in *Babesia rodhaini* infections, 71
Blood flow, renal, effect of noradrenaline on, 431
Blood plasma, substance in, causing slow contraction of guinea-pig gut *in vitro*, 93
———, and serum, smooth-muscle stimulants from, 111
Blood pressure, effect of 5-hydroxytryptamine on, in dogs, 44
———, role in ventricular arrhythmia production, 132
Blood sugar, effect of thiopentone on, 458
Brain, fish, Substance P-like polypeptide in, 323
———, Substance P from, 137
1-Bromo-1-chloro-2:2:2-trifluoroethane. *See* Fluothane
1-*p*-Bromodiphenylmethyl-4-methylpiperazine, anti-acetylcholine activity of, 273
(+)-2-Bromolysergic acid diethylamide, effect on central actions of 5-hydroxytryptamine, 175
———, effect on tidal-air reduction by 5-hydroxytryptamine, 289
———, effect on vasoconstrictor action of 5-hydroxytryptamine, 313
2-(*m*- and *p*-Bromophenoxy)ethyltrimethylammonium bromide, inactivation of amine oxidase by, 58
9-Butyl-2:7-diethoxycarbonylamino-10-methylphenanthridinium salt, trypanocidal activity of, 334
N-Butylnoradrenalone, hyperfibrinogenaemic action of, 99
Butyltriethylammonium bromide, depressor and mydriatic activities of, 282

C

288C53. *See* Ethylene-1-(ethyldimethylammonium)-2-(6:6-diphenylhex-5-enylammonium) diiodide
Carbonic anhydrase inhibitor, effect on gastric secretion, 263
Chloralose, antidiuretic activity in blood of animals anaesthetized with, effect of haemorrhage on, 236
Chlorambucil, effect on pregnancy in animals, 437
Chlorcyclizine, anti-acetylcholine activity of, 273
m- and *p*-Chlorobenzoylcholine, pharmacological actions of, 267
1-*m*-Chlorodiphenylmethyl-4-methylpiperazine, anti-acetylcholine activity of, 273
1-*p*-Chlorodiphenylmethyl-4-methylpiperazine. *See* Chlorcyclizine
1-*m*-Chlorodiphenylmethyl-4-methylpiperazine *N*-oxide, anti-acetylcholine activity of, 273
4-(2-Chloroethyl)morpholine, analgesic properties of, 27
2-(*p*-Chlorophenoxy)ethyltriethylammonium bromide, inactivation of amine oxidase by, 58
2-(*o*-, *m*- and *p*-Chlorophenoxy)ethyltrimethylammonium bromide, inactivation of amine oxidase by, 58
1-(*p*-Chlorophenylthien-2-ylmethyl)-4-methylpiperazine, anti-acetylcholine activity of, 273
Chloroquine phosphate, action in *Babesia rodhaini* infections, 71
Chlorpheniramine. *See* Chlorprophenpyridamine
Chlorpromazine, effect on vasoconstrictor action of adrenaline in man, 180
———, effect on vasoconstrictor action of noradrenaline, 318
———, pharmacological actions of, 339
Chlorprophenpyridamine, anti-acetylcholine activity of, 273
Chlortrimeton. *See* Chlorprophenpyridamine
Choline acetylase, brain, specificity of, 308
Choline *o*-chlorophenyl ether, inactivation of amine oxidase by, 58
Choline phenyl ethers, inactivation of amine oxidase by, 58
Choline *o*- and *p*-tolyl ether, inactivation of amine oxidase by, 58
Cholinesterases, activators of, 7
———, true, phosphorylated, chemical reactivation of, 295
Cinchocaine, local anaesthetic activity of, 104
Cocaine, local anaesthetic activity of, 104
(−)-Cocaine, effect on cholinesterase activity, 7
Colchicine, effect on pregnancy in animals, 437
Compound 48/80, effect on amine release from mast cells in rat, 411
———, effect on mast cell and histamine contents of guinea-pig tissues, 304
———, release of 5-hydroxytryptamine by, 202
Congasin. *See* Bis-4-amino-2-methylquinolyl-6-melamine
Cortisone, with and without isoniazid, effect on murine leprosy in mice, 326
Cysteine, protection against toxicity of Synkavit by, 128

D

Decamethonium, effect on cholinesterase activity, 7
Demecolcin, effect on pregnancy in animals, 437
Dextran sulphate, activation of rabbit serum-protease by, 107
———, inhibition of histamine release by, 357
Diacetyl monoxime, as antidote in anticholinesterase poisoning, 417
———, toxicity of, 424
Di(*p*-amidinophenyl)triazine diaceturate, action in *Babesia rodhaini* infections, 71
2:7-Diamino-9-*p*-aminophenyl-10-ethyl-, -methyl- and -propyl-phenanthridinium chloride, trypanocidal activities of, 330
2:7-Diamino-9-*p*-aminostyryl-10-ethyl- and -methyl-phenanthridinium bromide, trypanocidal activities of, 334

2:7-Diamino-9-benzyl-10-ethyl- and -methyl-phenanthridinium bromide, trypanocidal activities of, 330
2:7-Diamino-9-butyl- and -ethyl-10-methylphenanthridinium bromide, trypanocidal activities of, 334
2: 7-Diamino-9-butylphenanthridine, trypanocidal activity of, 334
2:7-Diamino-9-*p*-chlorophenyl-10-methylphenanthridinium chloride, trypanocidal activity of, 334
2:7-Diamino-10-(3-diethylamino- and -ethoxy-propyl)-9-phenylphenanthridinium methanesulphonate, trypanocidal activities of, 334
2:7-Diamino-9:10-diethylphenanthridinium bromide, trypanocidal activity of, 334
2:7-Diamino-9-*p*-dimethylaminostyryl-10-ethyl- and -methyl-phenanthridinium salts, trypanocidal activities of, 334
2:7-Diamino-9:10-dimethylphenanthridinium methanesulphonate, trypanocidal activity of, 330
2:7-Diamino-9-*p*-ethanesulphonylphenyl-10-ethyl- and -methyl-phenanthridinium salts, trypanocidal activities of, 334
2:7-Diamino-10-(2-ethoxy-, -hydroxy- and -methoxyethyl)-9-phenylphenanthridinium methanesulphonate, trypanocidal activities of, 334
2:7-Diamino-10-ethyl- and -methyl-9-*p*-methylphenylphenanthridinium bromide, trypanocidal activities of, 334
2:7-Diamino-10-ethyl- and -methyl-9-*p*-nitrophenylphenanthridinium chloride, trypanocidal activities of, 330
2:7-Diamino-10-ethyl- and -methyl-9-nonylphenanthridinium chloride, trypanocidal activities of, 334
2:7-Diamino-10-ethyl-9-methylphenanthridinium bromide, trypanocidal activity of, 330
2:7-Diamino-10-ethyl-9-phenylphenanthridinium bromide. *See* Ethidium bromide
2:7-Diamino-10-ethyl- and -methyl-9-styrylphenanthridinium methanesulphonate, trypanocidal activities of, 334
2:7-Diamino-10-ethyl- and -methyl-9-thien-2-ylphenanthridinium bromide, trypanocidal activities of, 330
2:7-Diamino-9-*p*-methoxyphenyl-10-methylphenanthridinium bromide, trypanocidal activity of, 334
2:7-Diamino-9-*p*-nitrophenyl-10-propylphenanthridinium chloride, trypanocidal activity of, 330
Di(*p*-aminophenoxy)alkanes, symmetrical, schistosomicidal activities of, effect of ring-substituents on, 375
———, unsymmetrical, schistosomicidal activities of, 367
2:7-Diamino-9-phenyl-10-propylphenanthridinium bromide, trypanocidal activity of, 330
Diamox. *See* Acetazoleamide
2-(2:5-Dibromophenoxy)ethyltrimethylammonium bromide, inactivation of amine oxidase by, 58
2-(2:4-Dichlorophenoxy)ethyltrimethylammonium bromide, inactivation of amine oxidase by, 58
2:7-Diethoxycarbonylamino-10-(3-diethylaminopropyl)-9-phenylphenanthridinium chloride, trypanocidal activity of, 334
2:7-Diethoxycarbonylamino-9:10-di-ethyl- and -methyl-phenanthridinium salts, trypanocidal activities of, 334
2:7-Diethoxycarbonylamino-10 (or 9)-ethyl-9 (or 10)-methylphenanthridinium salts, trypanocidal activities of, 334
Diethyl disulphide, antituberculous activity of, 351
Diethyl ethylenebis(*N*-4-phenylpiperidine-4-carboxylate), analgesic properties of, 27
5-(4-Diethylamino-2-hydroxypropylamino)-2:3-dimethoxy-8-nitroacridine, antiviral activities of, 220
5-(4-Diethylamino-1-methylbutylamino)-2:3-dimethoxy-8-nitroacridine, antiviral activities of, 220
5-(4-Diethylamino-1-methylbutylamino)-3-methoxy-8-nitroacridine, antiviral activities of, 220
Diethyldithiol*iso*phthalate, antituberculous activity of, 351
1:3-Di(hydroxyimino)acetone. *See* Di*iso*nitrosoacetone
Dimethanesulphonyloxyalkanes, effect on pregnancy in animals, 437
5-Dimethylamino-3-ethyl-1:2-dimethylindole, effect on central actions of 5-hydroxytryptamine, 175
Dimethyl-2-(*p*-tolyloxy)ethylamine hydrobromide, inactivation of amine oxidase by, 58
Dimethyl-3-(*p*-tolyloxy)propylamine hydrobromide, inactivation of amine oxidase by, 58
Dimidium bromide, trypanocidal activity of, 330
Di*iso*nitrosoacetone, toxicity of, 424
1-Diphenylmethyl-4-methylpiperazine, anti-acetylcholine activity of, 273
Dyflos poisoning, oximes and hydroxamic acids as antidotes in, 417

E

Ecolid. *See* Ethylene-1-trimethylammonium-2-(4:5:6:7-tetrachloro-*N*-methyl*iso*indolinium) dichloride
Eimeria tenella, action of pyrimethamine and sulphadimidine on, 454
Encephalomyelitis, eastern equine, antiviral activity of acridines in, in mice, 220
Ephedrine, hyperfibrinogenaemic action of, 99
Ergometrine, effect on central actions of 5-hydroxytryptamine, 175
———, effect on vasoconstrictor action of 5-hydroxytryptamine, 313
Ethanethiol. *See* Ethyl mercaptan
Ethanol, antidiuretic activity in blood of animals anaesthetized with, effect of haemorrhage on, 236
Ether, antidiuretic activity in blood of animals anaesthetized with, effect of haemorrhage on, 236
Ethidium bromide, action in *Babesia rodhaini* infections, 71
———, trypanocidal activity of, 330
Ethyl benzthiolate. *See* Ethylthiolbenzoate
Ethyl 1-(2-chloroethyl)-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 1-[2-{di(2-hydroxyethyl)amino}ethyl] -4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl dithiol*iso*phthalate. *See* Diethyldithiol*iso*phthalate
Ethyl 1-{2-(4-hydroxypiperidin-1-yl)ethyl}- 4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl mercaptan, antituberculous activity of, 351
Ethyl 1-(1-methyl-2-morpholinoethyl)-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 1-{2-(4-methylpiperidin-1-yl)ethyl} -4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 1-(2-morpholinoethyl)-4-phenylpiperidine-4-carboxylate, analgesic and other properties of, 27, 32
Ethyl 1-morpholinomethyl-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 1-(3-morpholinopropyl)-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 4-phenyl-1-(2-piperazin-1-ylethyl)piperidine-4-carboxylate, analgesic properties of, 27
Ethyl 4-phenylpiperidine-4-carboxylates, *N*-substituted, analgesic action of, 27
Ethyl 4-phenyl-1-(2-piperidinoethyl)piperidine-4-carboxylate, analgesic properties of, 27

Ethyl 4-phenyl-1-(2-pyrrolidin-1-ylethyl)piperidine-4-carboxylate, analgesic properties of, 27
Ethyl pyrophosphate poisoning, oximes and hydroxamic acids as antidotes in, 417
Ethyl 1-{2-(1 : 2 : 5 : 6-tetrahydropyrid-1-yl)ethyl}-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
Ethyl 1-{2-(2 : 3 : 4 : 5-tetrahydro-1 : 4-thiazin-4-yl)-ethyl}-4-phenylpiperidine-4-carboxylate, analgesic properties of, 27
N-Ethyl-*N'N'*-dimethyl-*N*-naphth-1- and -2-ylethylenediamine, pharmacological properties of, 1
N-Ethyl-*N'N'*-dimethyl-*N*-phenylethylenediamine, pharmacological properties of, 1
Ethylenebis(2-hydroxyethyldimethylammonium), depressor and mydriatic activities of, 282
Ethylenediamines, tetrasubstituted-*N*-derivatives of, pharmacological properties of, 1
Ethylenedi-4-morpholine, analgesic properties of, 27
Ethylene-1-(ethyldimethylammonium)-2-(6 : 6-diphenylhex-5-enylammonium) diiodide, depressor and mydriatic activities of, 282
Ethylene-1-trimethylammonium-2-(4 : 5 : 6 : 7-tetrachloro-*N*-methyl*iso*indolinium) dichloride, depressor and mydriatic activities of, 289
Ethylthiolbenzoate, antituberculous activity of, 351
Eyelid, inferior, of anaesthetized rat, as assay system, 147

F

m- and *p*-Fluorobenzoylcholine, pharmacological actions of, 267
1-*p*-Fluorodiphenylmethyl-4-methylpiperazine, anti-acetylcholine activity of, 273
Fluothane, anaesthetic action and pharmacological effects of, 394
Folic acid, effect on cholinesterase activity, 7
Frog, tree-, sperm-shedding response to gonadotrophins, 248

G

Ganglia, carbonyl receptors on, 267
———, superior cervical, effect of muscarine on, in cats, 442
———, sympathetic, action of 5-hydroxytryptamine on, 74
———, sympathetic and parasympathetic, action of ganglion-blocking agents on, 81
Ganglion-blocking action of methylenebisalkylsulphonium salts, 198
Ganglion-blocking agents, effects on sympathetic and parasympathetic ganglia in cat, 81
Gaplegin. *See* Hexamethylenebis(ethyldimethylammonium)
Gastric motility and secretion, effect of atropine and hexamethonium on, 231
Gastric secretion, effect of acetazoleamide on, 263
———, inhibition by quaternary atropine derivatives, 171
D-Glucosamine hydrochloride, effect on pregnancy in animals, 437
Glucose tolerance, effect of thiopentone on, 458
Gonadotrophins, sperm-shedding response of male toads and treefrogs to, 248

H

Haemorrhage, effect on release of neurohypophysial antidiuretic hormone in anaesthetized animals, 236
Hemlock water dropwort, pharmacological actions of, 225
Heparin, inhibition of histamine release by, 357
Hexamethonium, analogues of, depressor and mydriatic activities of, 282
———, depressor and mydriatic activities of, 282
———, effect on cholinesterase activity, 7
———, effect on sympathetic and parasympathetic ganglia in cat, 81
———, with atropine, effect on gastric motility and secretion, 231
Hexamethylenebisalkylsulphonium salts, ganglion-blocking action of, 198
Hexamethylenebis(dialkylamine) salts, ganglion-blocking action of, 198
Hexamethylenebis(*NN*-diethylanilinium), depressor and mydriatic activities of, 282
Hexamethylenebis(ethyldimethylammonium), depressor and mydriatic activities of, 282
Hexamethylenebis(2-hydroxyethyldimethylammonium), depressor and mydriatic activities of, 282
Hexamethylenebis(*N*-methylmorpholinium), depressor and mydriatic activities of, 282
4-(2-*cyclo*Hexylethyl)morpholine, analgesic properties of, 27
Histamine, metabolism in various species, 472
———, release of, by Compound 48/80 and reserpine in rat, 411
———, ———, inhibition by sodium salicylate and other compounds, 357
———, subcutaneous, urinary excretion of, 462
———, urinary excretion of, effect of enzyme inhibitors on, in man, 467
Histamine liberators, release of 5-hydroxytryptamine by, 202
Histidine, effect on cholinesterase activity, 7
Histidine decarboxylase, mammalian, 119
Hydroxamic acids, as antidotes in anticholinesterase poisoning, 417
Hydroxyiminoacetone. *See* Mono*iso*nitrosoacetone
3-Hydroxy-2-phenylcinchoninic acid, inhibition of histamine release by, 357
2- and 4-Hydroxy*iso*phthalic acid, analgesic and antipyretic properties of, 20
4-{2-(4-Hydroxypiperidin-1-yl)ethyl}morpholine, analgesic properties of, 27
5-Hydroxytryptamine, action on nictitating membrane and superior cervical ganglion in cat, 74
———, and its antagonists, effect on central nervous system, 175
———, ———, effect on tidal air, 289
———, antidiuretic activity in dogs, 35
———, biosynthesis of, 88
———, effect on blood pressure in dogs, 44
———, effect on ureter in dogs, 44, 50
———, estimation in presence of adrenaline, 66
———, in nettle sting, 186
———, release by Compound 48/80 and reserpine in rat, 411
———, release by histamine liberators, 202
———, vasoconstrictor action of, effect of lysergic acid derivatives on, 313
Hydroxytyramine, slow fibrinogenaemic action of, 99
(+)-, (−)- and (±)-Hyoscyamine, effect on cholinesterase activity, 7

I

Intestine, fish, Substance P-like polypeptide in, 323
———, Substance P from, 137
Iproniazid, effect on urinary excretion of free histamine in man, 467

Isoniazid, effect on experimental corneal tuberculosis, 190
———, effect on urinary excretion of free histamine in man, 467
———, with and without cortisone, effect on murine leprosy in mice, 326
Isoprenaline. *See N-iso*Propylnoradrenaline

K

α-Keto oximes, toxicities of, 424

L

Leprosy, murine, effect of cortisone and isoniazid on, in mice, 326
Lidocaine, local anaesthetic activity of, 104
Lignocaine. *See* Lidocaine
Liver, human and mammalian, inactivation of adrenaline and noradrenaline by, 52
Lymphogranuloma venereum, antiviral activity of acridines in, in chick embryos, 220
(+)-Lysergic acid diethylamide, effect on central actions of 5-hydroxytryptamine, 175
———, effect on tidal-air reduction by 5-hydroxytryptamine, 289
———, effect on vasoconstrictor action of 5-hydroxytryptamine, 313

M

Mast cells, amine release from, effect of reserpine and Compound 48/80 on, in rat, 411
———, effect of Compound 48/80 on, in guinea-pig, 304
Mepacrine action in *Babesia rodhaini* infections, 71
———, antiviral metabolites of, in mouse liver, 215
———, distribution in animal organs and liver-cell components, 209
Mephenesin, effect on cholinesterase activity, 7
6-Mercaptopurine, effect on pregnancy in animals, 437
Methadone, effect on central actions of 5-hydroxytryptamine, 175
p-Methoxybenzoylcholine, pharmacological actions of, 267
N-Methyladrenalone, hyperfibrinogenaemic action of, 99
Methylamphetamine, effect on central actions of 5-hydroxytryptamine, 175
3-Methyl-3-azapentamethylenebis(ethyldimethylammonium), depressor and mydriatic activities of, 282
m- and *p*-Methylbenzoylcholine, pharmacological actions of, 267
1-*p*-Methyldiphenylmethyl-4-methylpiperazine, anti-acetylcholine activity of, 273
N-Methylhydroxytyramine, slow hyperfibrinogenaemic action of, 99
Methylmedmain. *See* 5-Dimethylamino-3-ethyl-1 : 2-dimethylindole
Methylpentynol, mechanism of action on neuromuscular transmission in frog, 151
1-Methyl-1-phenyldithiobiuret, action in *Babesia rodhaini* infections, 71
1-Methyl-4-phenylthen-2-ylaminopiperidine, effect on tidal-air reduction by 5-hydroxytryptamine, 289
Mono*iso*nitrosoacetone, as antidote in anticholinesterase poisoning, 417
———, toxicity of, 424
Morphine, effect on central actions of 5-hydroxytryptamine, 175
———, effect on cholinesterase activity, 7
———, release of 5-hydroxytryptamine by, 202
Morpholinoethylnorpethidine. *See* Ethyl 1-(2-morpholinoethyl)-4-phenylpiperidine-4-carboxylate
Muscarine, effect on superior cervical ganglia in cats, 442
Muscle, smooth, slow contracting effect of plasma and serum on, 111
Myleran, effect on pregnancy in animals, 437

N

Nalorphine, central respiratory action in decerebrate dogs, 15
———, effect on cholinesterase activity, 7
Nervous system, central, effect of 5-hydroxytryptamine and its antagonists on, 175
Nettle sting, 5-hydroxytryptamine in, 186
Neuromuscular transmission, action of methylpentynol and paraldehyde on, in frog, 151
Nicotinhydroxamic acid methiodide, reactivation of phosphorylated true cholinesterases by, 295
Nitroakridin 3582. *See* 5-(4-Diethylamino-2-hydroxypropylamino)-2 : 3-dimethoxy-8-nitroacridine
m-Nitrobenzoylcholine, pharmacological actions of, 267
Noradrenaline, effect on respiration, 346
———, effect on ureter in dogs, 44
———, effect on urine and renal blood flow, 431
———, formation during splanchnic stimulation of adrenals in cat, 449
———, inactivation by liver *in vitro*, 52
———, slow hyperfibrinogenaemic action of, 99
———, vasoconstrictor action of, effect of chlorpromazine on, 318
Noradrenalone, hyperfibrinogenaemic action of, 99
Nucleotoxic substances, effect on pregnancy in animals, 437

O

Oenanthe crocata, pharmacological actions of, 225
Oenanthotoxin, pharmacological actions of, 225
Oximes, as antidotes in anticholinesterase poisoning, 417

P

Pamaquin naphthoate, action in *Babesia rodhaini* infections, 71
Paraldehyde, mechanism of action on neuromuscular transmission in frog, 151
Pendiomide, action on sympathetic and parasympathetic ganglia in cat, 81
———, depressor and mydriatic activities of, 282
Pentamethonium, action on sympathetic and parasympathetic ganglia in cat, 81
Pentamethylenebis(dimethylpropylammonium), depressor and mydriatic activities of, 282
Pentamethylenebis(*N*-ethylpiperidinium), depressor and mydriatic activities of, 282
Pentamethylenebis(2-hydroxyethyldimethylammonium), depressor and mydriatic activities of, 282
Pentamethylenebis(*N*-methylmorpholinium), depressor and mydriatic activities of, 282
Pentobarbitone, antidiuretic activity in blood of animals anaesthetized with, effect of haemorrhage on, 236
Pentolinium, depressor and mydriatic activities of, 282
Phenacemide, anticonvulsant activity of, effect of *Rauwolfia* alkaloids on, 141
Phenanthridine compounds, trypanocidal activity of, 330, 334
Phenidium chloride, trypanocidal activity of, 330
Pheniramine. *See* Prophenpyridamine
Phenylephrine, slow hyperfibrinogenaemic action of, 99
Phenytoin sodium, anticonvulsant activity of, effect of *Rauwolfia* alkaloids on, 141
Pholedrine, slow hyperfibrinogenaemic action of, 99
Pitressin, antidiuretic activity of, 245

Pituitary preparations, posterior, antidiuretic activity of, 245
———, ———, effect on ureter in dogs, 44
Pituitrin, antidiuretic activity of, 245
Plasma. *See* Blood plasma
Podophyllotoxin, effect on pregnancy in animals, 437
Polypeptide, Substance P-like, in fish brain and intestine, 323
Pregnancy, effect of neurotoxic substances on, in animals, 437
Procaine, local anaesthetic activity of, 104
(+)-, (−)- and (±)-Procyclidine ethiodide, hydrochloride and methiodide, effect on cholinesterase activity, 7
Proguanil acetate, action in *Babesia rodhaini* infections, 71
Propamidine, release of 5-hydroxytryptamine by, 202
Prophenpyridamine, anti-acetylcholine activity of, 273
*iso*Propyl methylphosphonofluoridate. *See* Sarin
*N-iso*Propylnoradrenaline, slow hyperfibrinogenaemic action of, 99
Protease, serum-, activation by dextran sulphate in rabbit, 107
Pseudocholinesterase, activators of, 7
Psittacosis, antiviral activity of acridines in, in mice, 220
Pyridine-2-aldoxime methiodide, reactivation of phosphorylated true cholinesterases by, 295
Pyrimethamine, action in *Babesia rodhaini* infections, 71
———, with sulphadimidine, mode of action on *Eimeria tenella*, 454

Q

Quinapyramine sulphate. *See* Antrycide methyl sulphate

R

RD 1660. *See* 1-Methyl-1-phenyldithiobiuret
2325 RP. *See N*-Ethyl-*N'N'*-dimethyl-*N*-phenylethylenediamine
2339 RP. *See N*-Benzyl-*N'N'*-dimethyl-*N*-phenylethylenediamine
Rauwolfia alkaloids, effect on anticonvulsant action of anti-epileptic drugs, 141
Receptor theory, modified, 379
Reserpine, effect on amine release from mast cells in rat, 411
———, effect on anticonvulsant action of anti-epileptic drugs, 141
———, effect on central actions of 5-hydroxytryptamine, 175
Respiration, effect of adrenaline and noradrenaline on, 346
———, effect of 5-hydroxytryptamine and its antagonists on, 289
———, effect of intravenous hydroxybenzoates on, 364
———, stimulatory action of nalorphine on, mechanism of, 15
Rift Valley fever, antiviral activity of acridines in, in mice, 220

S

SC–1919. *See* Butyltriethylammonium bromide
Su–3088. *See* Ethylene-1-trimethylammonium-2-(4 : 5 : 6 : 7-tetrachloro-*N*-methyl*iso*indolinium) dichloride
Salicylates, intravenous, hyperpnoea due to, 364
Sandosten. *See* 1-Methyl-4-phenylthen-2-ylaminopiperidine
Sarin poisoning, oximes and hydroxamic acids as antidotes in, 417
Schistosomicidal activities, of *p*-aminophenoxyalkanes, 367
Schistosomicidal activities, of *p*-aminophenoxy- and di(*p*-aminophenoxy)-alkanes, effect of ring-substituents on, 375
Sodium benzoate, inhibition of histamine release by, 357
Sodium chloride, inhibition of histamine release by, 357
Sodium *S*-ethyl thiosulphate, antituberculous activity of, 351
Sodium *o*-, *m*- and *p*-hydroxybenzoates, intravenous, effect on respiration, 364
Sodium salicylate, inhibition of histamine release by, 357
Spinal cord, perfused, frog's, experiments with, 161
Stylomycin, action in *Babesia rodhaini* infections, 71
Sulphadimidine, with pyrimethamine, mode of action on *Eimeria tenella*, 454
Substance P, from brain and intestine, 137
Suramin, action in *Babesia rodhaini* infections, 71
Synkavit, protection by cysteine against acute toxicity of, 128

T

Tartar emetic, action in *Babesia rodhaini* infections, 71
Tetraethylammonium, action on sympathetic and parasympathetic ganglia in cat, 81
Tetramethylammonium, action on sympathetic and parasympathetic ganglia in cat, 81
Tetramethylenebis(diethyl-2-hydroxyethylammonium), depressor and mydriatic activities of, 282
Tetrasodium 2-methyl-1 : 4-naphthaquinone diphosphate. *See* Synkavit
Thiambutene, effect on cholinesterase activity, 7
Thiol-carboxylic acids, ethyl esters of, antituberculous activities of, 351
Thiopentone, effect on blood sugar and glucose tolerance, 458
Toad, male, sperm-shedding response to gonadotrophins, 248
Tolazoline, effect on hyperfibrinogenaemic action of adrenaline, 99
Toxic substances, estimation of safe dose of, 156
(+)-, (−)- and (±)-Tricyclamol, effect on cholinesterase activity, 7
Triethylenemelamine, effect on pregnancy in animals, 437
NN'N'-Triethyl-*N*-naphth-1- and -2-ylethylenediamine, pharmacological properties of, 1
Triethyl-2-(*p*-tolyloxy)ethylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl colchicinic acid methyl ether, effect on pregnancy in animals, 437
Trimethyl-2-(dimethylphenoxy)ethylammonium bromides, inactivation of amine oxidase by, 58
Trimethylene-1-(diethylmethylammonium)-3-(2 : 7-diamino-9-phenyl-10-phenanthridinium) diiodide, trypanocidal activity, 334
Trimethyl-2-(5-methyl-2-*iso*propyl)ethylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl-2-phenoxyethylammonium, inactivation of amine oxidase by, 58
Trimethyl-3-phenoxypropylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl-3-phenylpropylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl-2-*o*-, -*m*- and -*p*-tolyloxyethylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl-3-*p*-tolyloxypropylammonium bromide, inactivation of amine oxidase by, 58
Trimethyl-2-(2 : 4 : 6-trimethylphenoxy)ethylammonium bromide, inactivation of amine oxidase by, 58
Trimeton. *See* Prophenpyridamine

Troxidone, anticonvulsant activity of, effect of *Rauwolfia* alkaloids on, 141
Tuberculosis, chemotherapy of, ethyl mercaptan and related compounds in, 351
———, corneal, experimental, effect of isoniazid on, 190
Tubocurarine, mode of action of, 428
Trypanocidal action of phenanthridine compounds, 330, 334
Trypanocides, action in *Babesia rodhaini* infections, 71
Tryparsamide, action in *Babesia rodhaini* infections, 71
Tyramine, hyperfibrinogenaemic action of, 99

U

Ureter, effect of adrenaline, noradrenaline and posterior pituitary extracts on, in dogs, 44
———, effect of 5-hydroxytryptamine on, in dogs, 44, 50
Urethane, antidiuretic activity in blood of animals anaesthetized with, effect of haemorrhage on, 236
Urine, flow of, effect of noradrenaline on, 431
Urtica dioica sting, 5-hydroxytryptamine in, 186

V

Ventricular arrhythmia, role of blood pressure in production of, 132
Vinylidene-1-(2:7-diamino-10-methyl-9-phenanthridinium)-2-(*p*-trimethylanilinium) di(methanesulphonate), trypanocidal activity of, 334
Vinylidene-1-(2:7-diamino-10-ethyl-9-phenanthridinium)-2-(*p*-ethyldimethylanilinium) dichloride, trypanocidal activity of, 334
Vinylidene-1-(2:7-diethoxycarbonylamino-10-ethyl-9-phenanthridinium)-2-(*p*-ethyldimethylanilinium), trypanocidal activity of, 334
Vinylidene-1-(2:7-diethoxycarbonylamino-10-methyl-9-, phenanthridinium)-2-(*p*-trimethylanilinium), trypanocidal activity of, 334

X

p-Xylylenebis(2-hydroxyethyldimethylammonium), depressor and mydriatic activities of, 282
p-Xylylenebis(*N*-methylmorpholinium), depressor and mydriatic activities of, 282
p-Xylylenebis(trimethylammonium), depressor and mydriatic activities of, 282

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CONTENTS

PAGE

Studies in the Chemotherapy of Tuberculosis: Ethyl Mercaptan and Related Compounds. G. E. DAVIES, G. W. DRIVER, E. HOGGARTH, A. R. MARTIN, M. F. C. PAIGE, F. L. ROSE, and B. R. WILSON 351

Inhibition of Histamine Release by Sodium Salicylate and Other Compounds. C. G. HAINING 357

The Hyperpnoea Produced by Intravenous Administration of Salicylates. J. B. COCHRAN and A. G. RAMSAY 364

The Activity of *p*-Aminophenoxyalkane Derivatives against *Schistosoma mansoni*. A. G. CALDWELL and O. D. STANDEN 367

Effect of Ring-substituents on the Activity of Diphenoxyalkanes against *Schistosoma mansoni*. O. D. STANDEN and L. P. WALLS 375

A Modification of Receptor Theory. R. P. STEPHENSON 379

The Action of Fluothane—A New Volatile Anaesthetic. J. RAVENTÓS 394

The Effects of Reserpine and Compound 48/80 on the Release of Amines from the Mast Cells of Rats. B. K. BHATTACHARYA and G. P. LEWIS 411

Oximes and Hydroxamic Acids as Antidotes in Anticholinesterase Poisoning. BERYL M. ASKEW 417

The Nature of the Toxicity of 2-Oxo-oximes. BERYL M. ASKEW, D. R. DAVIES, A. L. GREEN, and R. HOLMES 424

On the Mode of Action of Tubocurarine. M. BRZIN and A. O. ŽUPANČIĆ 428

Effect of Noradrenaline on Urine and Renal Blood Flow. F. G. W. MARSON 431

The Action of Some Nucleotoxic Substances on Pregnancy. K. DIDCOCK, D. JACKSON, and J. M. ROBSON 437

The Effect of Muscarine on Perfused Superior Cervical Ganglia of Cats. N. AMBACHE, W. L. M. PERRY, and P. A. ROBERTSON 442

Formation of Catechol Amines During Splanchnic Stimulation of the Adrenal Gland of the Cat. W. C. HOLLAND and H. J. SCHÜMANN 449

The Mode of Action of a Mixture of Pyrimethamine and Sulphadimidine on *Eimeria tenella*. L. P. JOYNER and S. B. KENDALL 454

Effect of Thiopentone on Blood Sugar and Glucose Tolerance. JOHN W. DUNDEE 458

The Urinary Excretion of Subcutaneously Injected Histamine. ROSS G. MITCHELL 462

The Effect of Enzyme Inhibitors on the Excretion of Free Histamine in Human Urine. ROSS G. MITCHELL 467

The Metabolism of Histamine in Various Species. RICHARD W. SCHAYER 472

Index to Volume 11 474

5577

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